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FRESHWATER CYANOBACTERIA (BLUE-GREEN ALGAE) TOXINS: ISOLATION AND CHARACTERIZATION

ANNUAL/FINAL REPORT

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May 1, 1990

SUPPORTED BY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MARYLAND 21702-5012

CONTRACT NO. DAMD17-87-C-7019

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; Distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Wright State University, Dept. of Biological Sciences; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Dayton, OH 45435	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-87-C-7019
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21702-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62770A	3M1-62770A871	AA	378

11. TITLE (Include Security Classification)

Freshwater Cyanobacteria (blue-green algae) Toxins: Isolation and Characterization

12. PERSONAL AUTHOR(S)

Wayne W. Carmichael, William Evans, Ann Kaup

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final Report	FROM 11/1/86 TO 4/30/90	1990 May 1	70

16. SUPPLEMENTARY NOTATION

Annual covers the period November 1, 1988 - April 30, 1990

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	13		RA I; Blue Green Algae; BW; Cyanobacteria; Toxins, Bioreactor Technology; Biotoxins
06	01		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Freshwater cyanobacteria (blue-green algae) are capable of producing several physiologically distinct toxins. These toxins are produced by strains of the bloom-forming species Anabaena flos-aquae, Microcystis aeruginosa, Aphanizomenon flos-aquae, Oscillatoria agardhii and Nodularia spumigena. Work carried out under this contract covered the following areas with these biotoxin-producing microorganisms: 1) Culture, using batch and semicontinuous culture methods, selected neuro- and hepatotoxin-producing strains of freshwater cyanobacteria. 2) Extract and purify the toxins using organic extraction followed by gel and ion-exchange column filtration (both standard and high performance liquid chromatography (HPLC). 3) Provide USAMRIID with selected purified hepatotoxic peptides and neurotoxic alkaloids. 4) Selected studies on optimization of culture conditions for toxic production and storage of toxic strains. 5) Continue collaborative studies in other areas of the U.S. and the world to isolate and compare freshwater/marine cyanobacteria toxins so that common methods of detection/decontamination can be developed.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY CONTENTS

This annual/final report covers work completed on "Freshwater Cyanobacteria (blue-green algae) Toxins: Isolation and Characterization". The first part of the report updates review material on toxins of freshwater cyanobacteria. The second part details studies covered under this contract as described in the contract workscope. The workscope areas include: 1) Development of culture methods of neuro- and hepatotoxin producing strains of freshwater cyanobacteria. This work has centered on implementation of fermenter systems designed for semi-continuous harvesting of algal cells, in addition to optimization of culture conditions for control of toxin production. 2) Extraction, purification and analysis of neurotoxins and hepatotoxins. This work has centered on purification and analysis of cyclic peptide toxins of Microcystis aeruginosa and Nodularia spumigena, and the neurotoxin ANTX-A(S) from Anabaena flos-aquae. 3) Toxicology work has involved the isolation and purification of the organophosphate anti-cholinesterase compound called anatoxin-a(s). 4) Collaborative studies to investigate new occurrences of toxic blue-green algae and to isolate, culture, and examine new toxic species. This work has resulted in the examination and isolation of new toxic isolates of Nostoc sp. from Finland, Oscillatoria sp. from Norway, Microcystis aeruginosa from China and Anabaena flos-aquae from Canada.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council. (DHEW Publication No. (NIH) 86-23, Revised 1985.)

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A. REVIEW UPDATE OF CYANOBACTERIA TOXINS

1. INTRODUCTION

Reports of toxic algae in the freshwater environment are almost exclusively caused by strains of species that are members of the division Cyanophyta, commonly called blue-green algae or cyanobacteria. Although cyanobacteria are found in almost any environment ranging from hot springs to Antarctic soils, known toxic members are mostly planktonic. Published accounts of field poisonings by cyanobacteria are known since the late 19th century (Francis, 1878). These reports describe sickness and death of livestock, pets, and wildlife following ingestion of water containing toxic algae cells or the toxin released by the aging cells. Recent reviews of these poisonings and the toxins of freshwater cyanobacteria are given by Carmichael (1981, 1986, 1988, 1989), Carmichael et al. (1990), Codd and Bell (1985), and Gorham and Carmichael (1988).

While about 12 genera have been implicated in cyanobacteria poisonings only toxins from Anabaena, Aphanizomenon, Microcystis, Nodularia, Nostoc, and Oscillatoria have been isolated, at least partially chemically defined and the toxins studied for their mode of action. In addition to the acute lethal toxins, some cyanobacteria produce potent cytotoxins. These secondary chemicals are not considered here but the reader is referred to papers by Barchi et al. (1983, 1984); Carmichael (1988); Moore et al. (1984, 1986); Mason et al. (1982) and Gleason and Paulson (1984) for further discussion of these compounds. These cytotoxins are also listed in Table 2.

Economic losses related to freshwater cyanobacterial toxins are the result of contact with or consumption of water containing toxin and/or toxic cells. These toxins are water-soluble and temperature-stable. They are either released by the cyanobacterial cell or loosely bound so that changes in cell permeability or age allow their release into the environment. Lethal and sublethal amounts of these toxins become available to animals during periods of heavy cell growth, termed "waterblooms," especially when the waterbloom accumulates on the surface, inshore, where animals are watering. Waterblooms can occur wherever proper conditions for growth, including irradiance, temperature, neutral or alkaline conditions, and nutrients are found. The increasing eutrophication of water supplies from urban and agricultural sources, which raises mineral nutrient levels, has increased the occurrence and intensity of these annual blooms. It should be noted that although there are several bloom-forming genera of cyanobacteria those that occur most often are also those that can produce toxins. Known occurrences of toxic cyanobacteria in water supplies (Table 1), include Canada (four provinces, Europe (12 countries), United States (20 states), USSR, Australia, India, Bangladesh, South Africa, Israel, Japan, New Zealand, Argentina, Chile and the Peoples Republic of China (Skulberg, et al., 1984; Carmichael et al., 1985, Gorham and Carmichael, 1988). Not all

blooms of a toxigenic species produce toxins, however, and it is not possible to tell by microscopic examination of the cells whether they are toxic. Environmental conditions that favor bloom formation include (1) moderate to high levels of nutrients, especially phosphorus and nitrate or ammonia, (2) water temperatures between 15 and 30°C, and (3) a pH between 6 and 9 or higher (Skulberg et al., 1984). The economic impact from toxic freshwater cyanobacteria include the costs incurred from deaths of domestic animals; allergic and gastrointestinal problems after human contact with water blooms (including lost income from recreational areas); and increased expense for the detection and removal of taste, odor, and toxins (although no approved method yet exists for removal of toxins, activated carbon has been tried in certain areas). This section summarizes the neurotoxins and hepatotoxins of fresh and brackish water cyanobacteria. A summary of these compounds is given in Table 2.

Table 1. Known Occurrences of Toxic Cyanobacteria in Fresh or Marine Water (updated from Gorham and Carmichael, 1988)

ARGENTINA	NEW ZEALAND
AUSTRALIA	OKINAWA (MARINE)
BANGLADESH	PEOPLES REPUBLIC OF CHINA
BERMUDA	SOUTH AFRICA
BRAZIL	
CANADA	U.S.A.
Alberta	California
Manitoba	Colorado
Ontario	Hawaii (marine)
Saskatchewan	Idaho
	Illinois
EUROPE	Iowa
	Michigan
Czechoslovakia	Minnesota
Denmark	Montana
East Germany	Nevada
Finland	New Hampshire
Great Britain	New Mexico
Hungary	New York
Netherlands	North Dakota
Norway	Oregon
Poland	Pennsylvania
Portugal	South Dakota
Sweden	Texas
West Germany	Washington
	Wisconsin
INDIA	
ISRAEL	U.S.S.R.
JAPAN	
	Ukraine

World map showing areas (darkened) where toxic freshwater cyanobacteria have been found.

Disease Related to Freshwater Algae Blooms

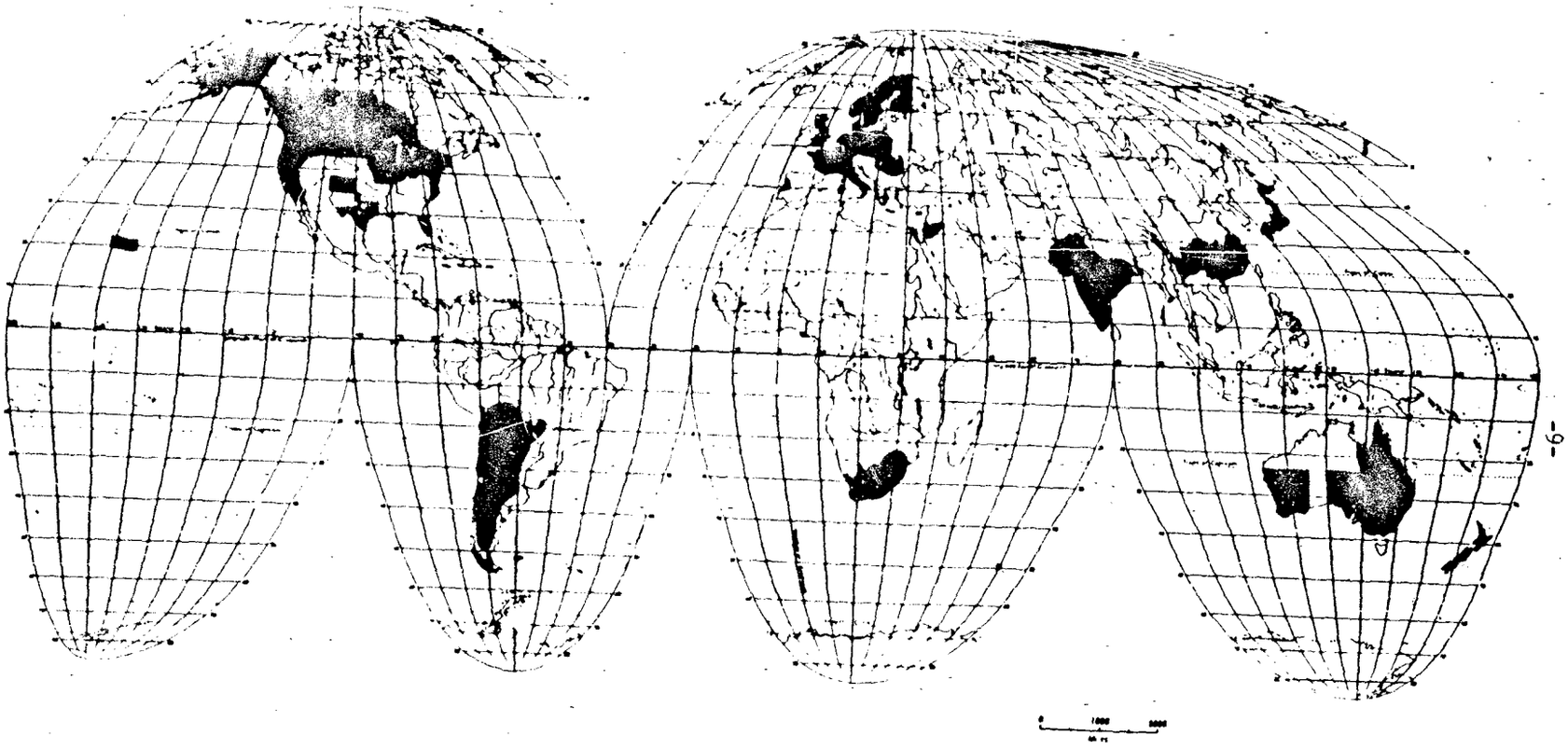


Table 2. Toxins of Freshwater Cyanobacteria

Species, strain, and source	Toxin term	Structure	LD_{50} μg/kg IP, mouse
Neurotoxins			
Anabaena flos-aquae	Anatoxin-A	Secondary amine alkaloid, MW 165	200
Strain NRC-44-1 (Canada, Saskatchewan)			
Strain NRC-525-17 (Canada, Saskatchewan)	Anatoxin-A(S)	Organophosphate alkaloid, MW 252	50
Aphanizomenon flos-aquae	Aphantoxin (neosaxitoxin)	Purine alkaloid MW 315 (neoSTX) MW 299 (STX)	10
Strain NH-1 & NH-5 (U.S., New Hampshire)	Aphantoxin II (saxitoxin)		
Hepatotoxins			
Anabaena flos-aquae	Microcystins[a]	Heptapeptides MW 994	50
Strain S-23-g-1 (Canada, Saskatchewan)			
Microcystis aeruginosa	Cyanoginosins[a]	Heptapeptides MW 909-1044	50
Strain WR-70 (=UV-010) (South Africa, Transvaal)			
(Waterbloom, Australia, New South Wales)	Cyanoginosin	Heptapeptide MW 1035	50
(Waterbloom, U.S., Wisconsin	Microcystin	Heptapeptide MW 994	50
Strain NRC-1(SS-17) (Canada, Ontario)	Microcystin	Heptapeptide MW 994	50
Strain 7820 (Scotland, Loch Balgaves)	Microcystin	Heptapeptide MW 994	50
(Waterbloom, Norway, Lake Akersvatn)	Microcystin	Heptapeptide MW 994	50
Microcystis aeruginosa	Microcystin	Heptapeptide MW 994 MW 1044	50
Strain M-228 (Japan, Tokyo)			

Microcystis aeruginosa	Cyanogenosin[a]	Heptapeptide MW 1039	not reported
Microcystis viridis	Cyanoviridin[a]	Heptapeptide MW 1039	not reported
Nodularia spumigena	Nodularin	Pentapeptide MW 824	30-50
Oscillatoria agardhii var. isothrix (Waterbloom, Norway, Lake Froylandsvatn)	Microcystins	Heptapeptides MW 1009	300-500
Oscillatoria agardhii var. (Waterbloom, Norway, Lake Kolbotnvatn)	Microcystins	Heptapeptides MW 1023	500-1000
Cytotoxins			
Scytonema pseudohofmanni Strain BC-1-2 (U.S., Hawaii)	Scytophycin A & B	Methylformamide A-MW 821; B-MW 819	650 (scytophycin B)
Scytonema hofmanni Strain UTEX-1581 (U.S., Texas)	Cyanobacterin	Chlorinated diaryllactone	not reported
Hapalosiphon fontinalis Strain V-3-1 (Marshall Islands)	Hapalindole A	Substituted indole alkaloid	not reported
Tolypothrix byssoidea	Tubercidin	Pyrrolopyrimidine	not reported
Oscillatoria acutissima Strain B-1 (U.S., Hawaii)	Acutiphycin	Macrolide	not reported

[a]See text for explanation of terminology.

2. NEUROTOXINS

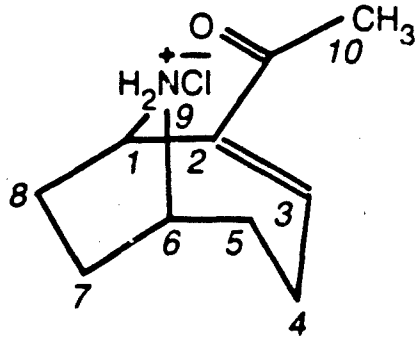
a. Anatoxins

Neurotoxins produced by filamentous Anabaena flos-aquae are called anatoxins (ANTX) (Carmichael and Gorham, 1978). Two anatoxins [ANTX-A and A(S)] are available for structure and function studies. ANTX-A from strain NRC-44-1 is the first toxin from a freshwater cyanobacteria to be chemically defined. It is the secondary amine, 2-acetyl-9-azabicyclo (4-2-1) non-2-ene (Huber, 1972; Devlin et al., 1977), molecular weight 166 daltons (Fig. 1). It has been synthesized through a ring expansion of cocaine (Campbell et al., 1977, 1979), from iminium salts (Bates and Rapoport, 1979; Peterson et al., 1984, 1985), from 4-cycloheptenone or tetrabromotricyclooctane (Danheiser et al., 1985) by construction of the azabicyclo ring from 9-methyl-9-azabicyclo [3.3.1] nonan-1-ol (Wiseman and Lee, 1986), and by starting with 9-methyl-9-aza[4.2.1] nonan-2-one (Lindgren et al., 1987).

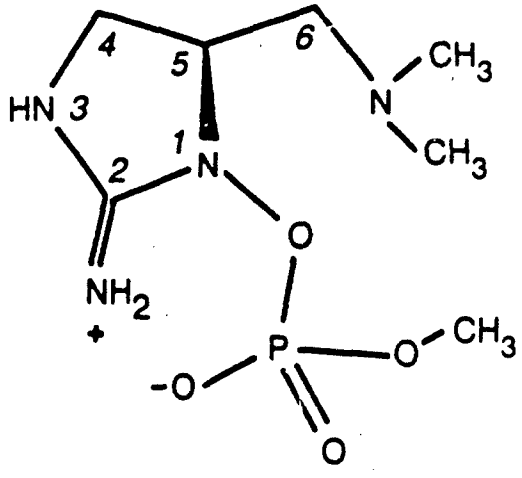
Fig. 1. (top left) Anatoxin-a (ANTX-A) hydrochloride. Produced by the freshwater filamentous cyanobacterium Anabaena flos-aquae NRC-44-1.

(top right) Anatoxin-a(s) (ANTX-A(s)). Produced by the freshwater filamentous cyanobacterium Anabaena flos-aquae NRC-525-17.

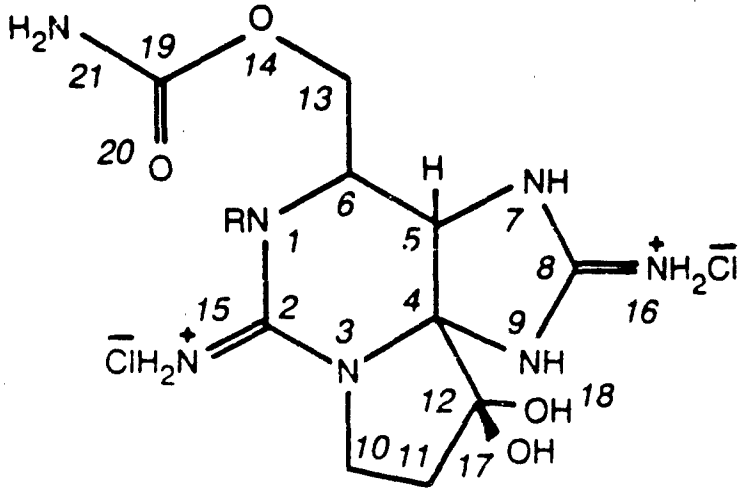
(bottom) Aphantoxin-I (neosaxitoxin) and Aphantoxin-II (saxitoxin) produced by certain strains of the filamentous cyanobacterium Aphanizomenon flos-aquae.



anatoxin - a hydrochloride
(m/z 165)
$C_{10}H_{15}NO$



anatoxin - a(s)
(m/z 252)
$C_7H_{17}N_4O_4P$



R = H; saxitoxin dihydrochloride
R = OH; neosaxitoxin dihydrochloride

ANTX-A is a potent, postsynaptic, depolarizing, neuromuscular blocking agent that affects both nicotinic and muscarinic acetylcholine (ACH) receptors at the ACH channel (Carmichael et al., 1979; Spivak et al., 1980, 1983; Aronstam and Witkop, 1981). Signs of poisoning in field reports for wild and domestic animals include staggering, muscle fasciculations, gasping, convulsions, and opisthotonos (birds). Death by respiratory arrest occurs within minutes to a few hours depending on species, dosage, and prior food consumption. The LD_{50} intraperitoneal (IP) mouse for purified toxin is about 200μg/kg body weight, with survival time of 4-7 min. This means that animals need to ingest only a few milliliters to a few liters of the toxic surface bloom to receive a lethal bolus (Carmichael and Gorham, 1977; Carmichael et al., 1977, Carmichael and Biggs, 1978).

Anatoxin-A(S) [ANTX-A(S)], produced by A. flos-aquae NRC-525-17, is different from ANTX-A. It produces opisthotonos in chicks, as does ANTX-A, but also causes viscous salivation [which gives the terminology its (S) label] and lachrymation in mice, chromodacryorrhea in rats, urinary incontinence, and defecation prior to death by respiratory arrest. Also observed is a dose-dependent fasciculation of limbs for 1-2 min after death. ANTX-A(S) has been purified by column chromatography and high-performance liquid chromatography (HPLC) (Carmichael and Mahmood, 1984), and its structure is given in Fig. 1 (Matsunaga et al. 1989). ANTX-A(S) is acid stable, unstable in basic conditions, has very low ultraviolet (uv) absorbance, gives a positive alkaloid test, and has a molecular weight of 252 daltons.

The LD_{50} IP mouse for ANTX-A(S) is about 20 μg/kg, over ten times more toxic than ANTX-A. At the LD_{50} the survival time for mice is 10-30 min. Mahmood and Carmichael (1986a) conclude that the toxicological and pharmacological signs of poisoning indicate excessive, cholinergic stimulation. Recent work by Mahmood and Carmichael (1987) and Hyde (1989, Ph.D. Thesis, Wright State University) shows that ANTX-A(S) is an irreversible anticholinesterase.

Mahmood and co-workers (1988) have identified ANTX-A(S) as the probably cause of death for five dogs, eight pups and two calves that ingested quantities of A. flos-aquae in Richmond Lake, South Dakota, in late summer 1985. At present all neurotoxic A. flos-aquae strains studied in the laboratory have come from North America. There are, however, some recent reports of neurotoxic Anabaena in Australia (Runnegar et al., 1988a), Japan and Scandinavia (M. Watanabe and O.M. Skulberg, personal communication; Sivonen et. al., 1989a). It seems likely that once they are looked for, neurotoxic Anabaena will be found in all the same geographic areas as other toxic cyanobacteria.

b. Aphantoxins

Occurrence of neurotoxins (aphantoxins) in the freshwater filamentous cyanobacterium Aphanizomenon flos-aquae was first demonstrated by Sawyer and co-workers (1968). All aphantoxins (APHTXS) studied to date have come from waterblooms and laboratory strains of nonfasciculate (non-flake-forming) Aph. flos-aquae that occurred in lakes and ponds of New Hampshire from 1966 through 1980. Toxic cells and extracts of Aph. flos-aquae were shown to be toxic to mice, fish, and waterfleas (Daphnia catawba) by Jakim and Gentile (1968). Chromatographic and pharmacological evidence established that APHTXS consist mainly of two neurotoxic alkaloids that strongly resembled saxitoxin (STX) and neosaxitoxin (neoSTX), the two primary toxins of red tide paralytic shellfish poisoning (PSP) (Sasner et al., 1984). The bloom material and toxic strain used in studies before 1980 came from collections made between 1960 and 1970. The more recent work on APHTXS has used two strains (NH-1 and NH-5) isolated by Carmichael in 1980 from a small pond near Durham, New Hampshire (Carmichael, 1982; Ikawa et al., 1982). These APHTXS, as well as neoSTX and STX, are fast-acting neurotoxins that inhibit nerve conduction by blocking sodium channels without affecting permeability to potassium, and transmembrane resting potential, or membrane resistance (Adelman et al., 1982). Mahmood and Carmichael (1986b), using the NH-5 strain showed that batch-cultured cells have a mouse IP LD_{50} of about 5 mg/kg. Each gram of lyophilized cells yields about 1.3 mg aphantoxin I (neosaxitoxin) and 0.1 mg aphantoxin II (saxitoxin) (Fig. 1). Also detected were three unstable neurotoxins that were not similar to any of the known paralytic shellfish poisons.

Shimizu and co-workers (1984) studied the biosynthesis of the STX analog neoSTX using Aph. flos-aquae NH-1. They were able to confirm its presence in strain NH-1 and to explain the biosynthetic pathway for this important group of secondary chemicals.

3. HEPATOTOXINS

Low-molecular-weight peptide toxins that affect the liver have been the predominant toxins involved in cases of animal poisonings due to cyanobacterial toxins (Schwimmer and Schwimmer, 1968; Carmichael, 1986, 1989; and Gorham and Carmichael, 1988). After almost 25 years of structure analysis on toxic peptides of the colonial bloom-forming cyanobacterium Microcystis aeruginosa, Botes and co-workers (1982a,b, 1986) and Santikarn and colleagues (1983) provided structure details on one of four toxins (designated toxin BE-4) produced by the South African M. aeruginosa strain WR70 (= UV-010). They concluded that it was monocyclic and contained three D-amino acids--alanine, erythro-β-methylaspartic acid, and glutamic acid, two L-amino acids--leucine and alanine--plus two unusual amino acids. These were N-methyldehydroalanine (Medha) and a nonpolar side chain of 20 carbon atoms that turned out to be a novel β-amino acid; 3-amino-

9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA). Based on fast atom bombardment mass spectrometry (FABMS) and nuclear magnetic resonance (NMR) studies, BE-4 toxin is now known to be a cyclic heptapeptide having a molecular weight of 909 daltons. Botes and co-workers (1985) also showed that the other three toxins of strain WR-70 all had the same D-amino acids and the two novel amino acids (Medha and ADDA). They differed in that the L-amino acids were leucine-arginine; tyrosine-arginine and tyrosine-alanine instead of leucine-alanine as in toxin BE-4. They were also able to show that the hepatotoxin isolated by Elleman and colleagues (1978) from water bloom material collected in Malpas Dam, New South Wales, Australia, contained the five characteristic amino acids plus the L-amino acid variants tyrosine-methionine.

Instead of calling the BE-4 toxin microcystin, as previous Microcystis toxins were called (Konst et al., 1965; Murthy and Capindale, 1970; Rabin and Darbre, 1975) and using alphabetical or numerical suffixes to indicate chromatographic elution order or structural differences, Botes (1986) proposed the generically derived designation cyanoginosin (CYGSN). This name, which indicates the cyanobacterial species (i.e. aeruginosa) origin, is followed by a two-letter suffix that indicates the identity and sequence of the two L-amino acids relative to the N-Me-dehydroalanyl-D-alanine bond. Thus toxin BE-4 was renamed cyanoginosin-LA since leucine and alanine are the L-amino acids.

Microcystin (MCYST) is the term given to the fast death factor (FDF) produced by M. aeruginosa strain NRC-1 and its daughter strain NRC-1 (SS-17) (Bishop et al., 1959; Konst et al., 1965). A definitive structure for the toxin of strain NRC-1 (SS-17) is not yet available but is known to be a peptide (MW 994) containing the variant L-amino acids leucine and arginine (Carmichael, unpublished). Krishnamurthy and co-workers (1986a,b) have shown that the toxin isolated from a waterbloom of M. aeruginosa collected in Lake Akersvatn, Norway (Berg et al., 1987), has a structure similar to that of MYCST from NRC-1 (SS-17) and CYGSN-LR. This toxin has also been found to be the main toxin produced by the Scottish strain of M. aeruginosa PCC-7820 and a Canadian A. flos-aquae strain S-23-g-1 (Krishnamurthy et al., 196 a,b). The identification of a peptide toxin from A. flos-aquae S-23-g-1 provides the first evidence that these hepatotoxins are produced by filamentous as well as coccoid cyanobacteria. A. flos-aquae S-23-g-1 and toxic M. aeruginosa from a waterbloom in Wisconsin also produced a second cyclic heptapeptide hepatotoxin, which has been found to have six of the same amino acids, that is, leucine-arginine, but has aspartic acid instead of β-methylaspartic acid (Krishnamurthy et al., 1986a).

The filamentous genus Oscillatoria has also been shown to produce a hepatotoxin (Ostensvik et al., 1981; Eriksson et al., 1987a). From water blooms of O. agardhii var and O. agardhii var. isothrix, two similar cyclic heptapeptides have been isolated. Both toxins have the variant L-amino acids arginine-arginine and

aspartic acid instead of β-methylaspartic acid. The toxin from O. agardhii var. isothrix also has dehydroalanine instead of methyldehydroalanine (Krishnamurthy et al., 1986b; Meriluoto et. al., 1989). More recently M. viridis (Kusumi et al., 1987) and M. aeruginosa (Painuly et al., 1988; Harada et al., 1988) have been shown to produce the cyclic heptapeptide with an arginine-arginine "L" amino acid variant. Harada et. al. (1990) showed that an ADDA isomer of MCYST-LR and -RR has very low toxicity, indicating biological activity of microcystin resides with the ADDA. In addition, Namikoshi et. al. (1989) described a total synthesis for ADDA making it possible to do more precise structure/function studies.

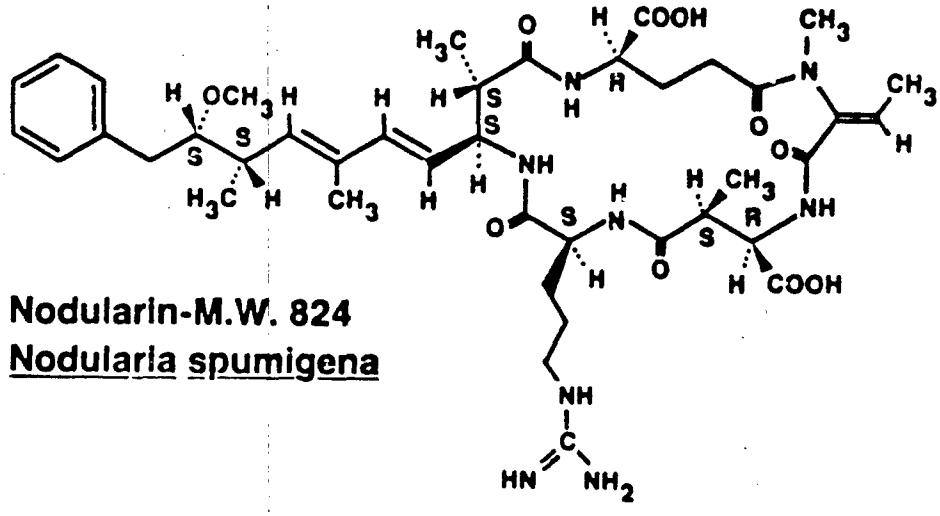
Nodularia spumigena has also been shown to produce a peptide with hepatotoxic activity. The more recent reports come from Australia (Main et al., 1977), the German Democratic Republic (Kalbe and Tiess, 1964), Denmark (Lindstrom, 1976), Sweden (Edler et al., 1985) and Finland (Eriksson et al., 1988a; Persson et al., 1984). Recently structure information on Nodularia toxin has been presented by Rinehart et. al. (1988) for waterbloom material collected in Lake Forsythe, New Zealand in 1984; by Carmichael and co-workers (1988) for a clonal isolate from Lake Ellesmere, New Zealand; by Eriksson and co-workers (1988) from waterbloom material collected in the Baltic Sea in 1986 and Runnegar and colleagues (1988b) for a field isolate from the Peel Inlet, Perth, Australia and Sivonen et. al. (1989b) for field material and laboratory isolation from the Baltic Sea. Structure work by these groups all indicate that the peptide is smaller than the heptapeptides toxins. Rinehart and co-workers (1988) showed that the toxin is a pentapeptide with a similar structure to the heptapeptides and containing β-methylaspartic acid, glutamic acid, arginine, N-methyl-dehydrobutyrine and ADDA (M.W. 824) (Fig. 2).

1. Mode of Action for Microcystins

The liver has always been reported as the organ that showed the greatest degree of histopathological change when animals are poisoned by these cyclic peptides. The molecular basis of action for these cyclic peptides is not yet understood but the cause of death from toxin and toxic cells administered to laboratory mice and rats is at least partially known and is concluded to be hypovolemic shock caused by interstitial hemorrhage into the liver (Theiss et al., 1988). This work with small animal models is currently being extended to larger animals in order to study the uptake, distribution, and metabolism of the toxins (Beasley et al., unpublished data). There is evidence to show from studies using ^{125}I-labeled CYGSN-YM (MCYST-YM) that the liver is the organ for both accumulation and excretion (Falconer et al., 1986; Runnegar et al., 1986a). Brooks and Codd (1987), using C^{14} labeled MCYST-LR, showed that seventy percent of the labeled toxin was localized in the mouse liver after 1 min following intraperitoneal injection of the toxin.

Studies at both the light and electron microscopic (EM) level of time-course histopathological changes in mouse liver show rapid and extensive centrilobular necrosis of the liver with loss of characteristic architecture of the hepatic cords.

Fig. 2 Structure of nodularin (NODLN) produced by Nodularia spumigena waterbloom from Lake Forsythe, New Zealand and clonal isolate L575 from Lake Ellesmere, New Zealand (Rinehart et al., 1988). It is also the same as produced by field material and laboratory cultures of N. spumigena from the Baltic Sea (Sivonen et. al., 1989b).



Sinusoid endothelial cells and then hepatocytes show extensive fragmentation and vesiculation of cell membranes (Runnegar and Falconer, 1981; Foxall and Sasner, 1981). Using microcystin-LR from M. aeruginosa strain PCC-7820, Dabholkar and Carmichael (1987) and Hooser et. al. (1990) found that at both lethal and sublethal toxin levels hepatocytes show progressive intracellular changes beginning at about 10 min postinjection. The most common response to lethal and sublethal injections is vesiculation of rough endoplasmic reticulum (RER), swollen mitochondria, and degranulation (partial or total loss of ribosomes from vesicles). The vesicles appear to form from dilated parts of RER by fragmentation or separation. Affected hepatocytes remain intact and do not lyse. Use of the isolated perfused rat liver to study the pathology of these toxins shows similar results to the in vivo work. Berg and co-workers (1988) used three structurally different cyclic heptapeptide hepatotoxins (MCYST-LR; desmethyl MCYST-RR and didesmethyl MCYST-RR). All three toxins had a similar effect on the perfused liver system although both "RR" toxins required higher concentrations (5-7x) to produce their effect. This was consistent with the lower toxicity of the "RR" toxins, which was about 500 and 1000 μg/kg i.p. mouse compared to 50 μg/kg for MCYST-LR.

In vitro studies on isolated cells including hepatocytes, erythrocytes, fibroblasts and alveolar cells continue to demonstrate the specificity of action that these toxins have for liver cells (Eriksson et al., 1987a, 1988b; Runnegar et al., 1987; Falconer and Runnegar, 1987; Eriksson et. al., 1989). This has led Aune and Berg (1987) to use isolated rat hepatocytes as a screen for detecting hepatotoxic waterblooms of cyanobacteria.

The cellular/molecular mechanism of action for these cyclic peptide toxins is now an area of active research in several laboratories. These peptides cause striking ultrastructural changes in isolated hepatocytes (Runnegar and Falconer, 1986b) including a decrease in the polymerization of actin. This effect of the cells cytoskeletal system continues to be investigated and recent work supports the idea that these toxins interact with the cells cytoskeletal system (Eriksson et al., 1987b; Falconer and Runnegar, 1987; Eriksson et. al., 1989). The apparent specificity of these toxins for liver cells is not clear although it has been suggested that the bile uptake system may be at least partly responsible for penetration of the toxin into the cell (Berg, et al., 1988).

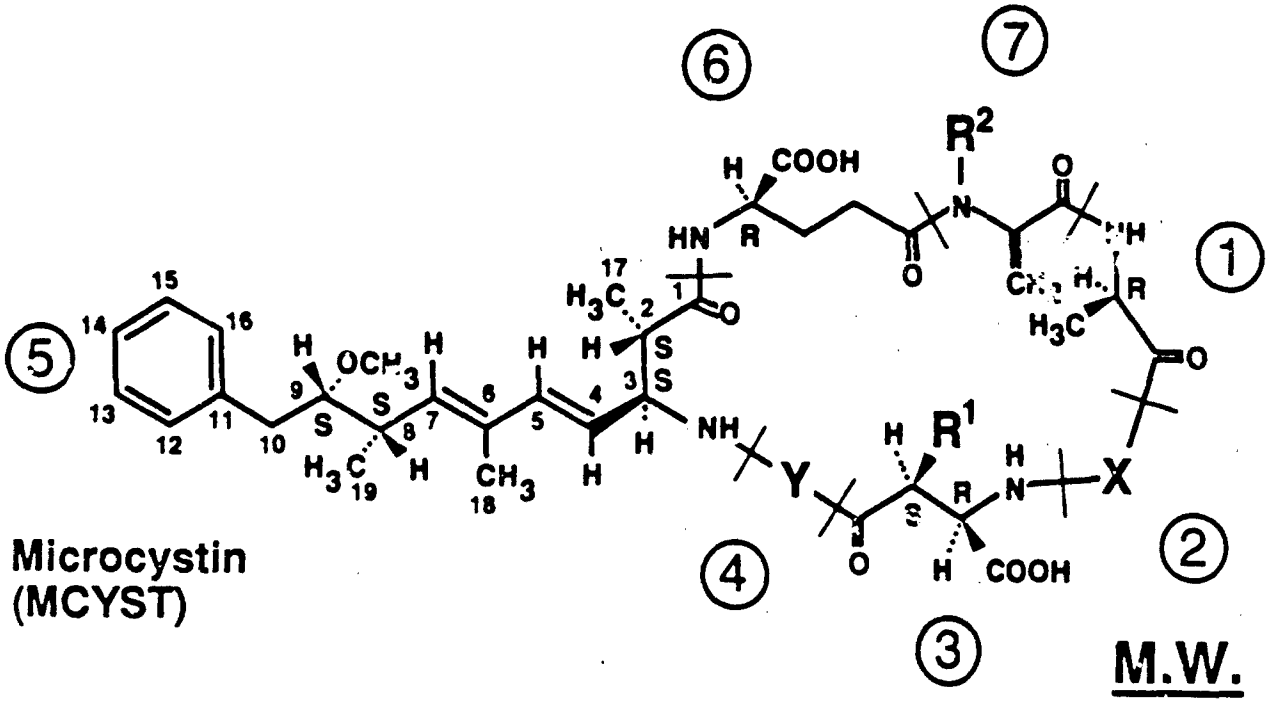
2. Naming the Cyclic Peptide Hepatotoxins

The hepatotoxins have been called Fast-Death Factor (Bishop et al., 1959), Microcystin (Konst et al., 1965), Cyanoginosin (Botes et al., 1986), Cyanoviridin (Kusumi et al., 1987) and Cyanogenosin (apparently a misspelling of cyanoginosin) (Painuly et al., 1988). Continued use of this multiple naming system will create confusion and misunderstanding as more is published on these cyclic peptides. A number of investigators doing research on these toxins have therefore proposed a system of nomenclature

based on the original term microcystin (MCYST) (Carmichael et al., 1988). Using this system the structures of known microcystins are given in Figs. 3 and 4. The distribution of microcystins in cyanobacteria shows some interesting patterns (Table 3). Most notable is that all of the methylated or demethylated homologues occur in genera other than Microcystis while Microcystis shows more variation with regard to the variant L-amino acids.

Fig. 3 Structure of known microcystins (refer also to Table 2).

Structure of seven microcystins varying only in L-amino acids and two microcystins with desmethyl portions of amino acids 3 and 7



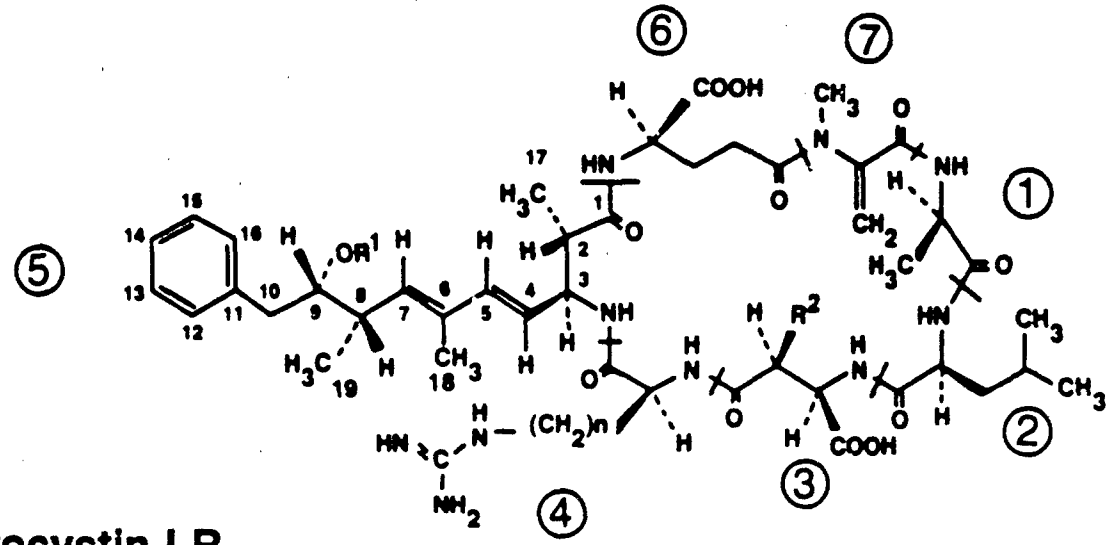
Microcystin (MCYST)

		M.W.
	MCYST - LA: X = Leu; R^1 = CH_3; Y = Ala; R^2 = CH_3	909
	MCYST - YA: X = Tyr; R^1 = CH_3; Y = Ala; R^2 = CH_3	959
	MCYST - LR: X = Leu; R^1 = CH_3; Y = Arg; R^2 = CH_3	994
	MCYST - FR: X = Phe; R^1 = CH_3; Y = Arg; R^1 = CH_3	1028
	MCYST - YM: X = Tyr; R^1 = CH_3; Y = Met; R^2 = CH_3	1035
	MCYST - RR: X = Arg; R^1 = CH_3; Y = Arg; R^2 = CH_3	1037
[D-Asp^3]	MCYST - RR: X = Arg; R^1 = H; Y = Arg; R^2 = CH_3	1023
[D-Asp^{3,7}]	MCYST - RR: X = Arg; R^1 = H; Y = Arg; R^2 = CH_3	1009
	MCYST - YR: X = Tyr; R^1 = CH_3; Y = Arg; R^2 = CH_3	1044

Fig. 4 Structure of known Microcystin-LR homologues.

**D-Asp = desmethyl aspartic acid
*ADMAdda = acetyl ADDA
*Har = homoarginine

* produced by Nostoc sp.
**produced by Anabaena and Nostoc strains.



Microcystin-LR and analogues

	M.W.
MCYST - LR:R^1 = CH_3; = R^2 = CH_3; n=3	994
[ADMAdda^5] - MCYST - LR:R^1 = $COCH_3$; R^2 = CH_3; n = 3	1022
[ADMAdda^5] - MCYST - LHar:R^1 = $COCH_3$; R^2 = CH_3; n = 4	1036
[D-Asp^3,ADMAdda^5] - MCYST - LR:R^1 = $COCH_3$; R^2 = H; n = 3	1008
[D-Asp^3,ADMAdda^5] - MCYST - LHar:R^1 = $COCH_3$; R^2 = H; n = 4	1022
[D-Asp^3] - MCYST - LR:R^1 = CH_3; R^2 = H; n = 3	980

TABLE 3

SOURCES OF MICROCYSTIN AND NODULARIN

Organism	Type of Microcystin
Microcystis aeruginosa	MCYST-LR, LA, YR, FR, YM, RR, LAba
Microcystis viridis	MCYST-RR, LR, YR, LA
Microcystis wesenbergii	MCYST-RR, LR (based upon mixed waterbloom samples)
Oscillatoria agardhii var. isothrix	MCYST-RR [D-Asp^{3}]-MCYST-RR [D-Asp^{3,7}]-MCYST-RR
Oscillatoria agardhii var. (red pigmented)	MCYST-RR [D-Asp^{7}]-MCYST-RR
Anabaena flos-aquae	MCYST-LR [D-Asp^{3}]-MCYST-LR
Nostoc sp.	MCYST-LR [ADMAdda^{5}]-MCYST-LR [ADMAdda^{5}]-MCYST-LHar [D-Asp^{3},ADMAdda^{5}]-ADDA-MCYST-LR [D-Asp^{3},ADMAdda^{5}]-MCYST-LHar [D-Asp^{3}]-MCYST-LR

Aphanizomenon and Coelosphaerium are reported to produce peptide hepatotoxins but specific ones have not been isolated.

Nodularia spumigena	Nodularin

B. WORKSCOPE (Experimental Programs)

1. Culture, Harvest, and Cell Yields of Toxic Blue-Green Algae (Cyanobacteria).

The following strains are batch and semi-continuously cultured for toxin production and for toxin analysis:

Anabaena flos-aquae strain	NRC-525-17
Aphanizomenon flos-aquae strain	NH-5-a
Microcystis aeruginosa strain	M-228
Microcystis aeruginosa strain	UV-027
Nodularia spumigena strain	L-575
Microcystis aeruginosa strain	PCC-7820 (nonaxenic)
Microcystis aeruginosa strain	PCC-7820 (axenic)
Anabaena flos-aquae strain	IG-20
Anabaena flos-aquae strain	44-1-s-30
Anabaena sp.	VS-1
Anabaena circinalis strain	IC-1

Anabaena flos-aquae strain 525-17-b-1-e is batch cultured at room temperature in (2) 200-liter tubs to provide material for extraction of Anatoxin-a(s). These fiberglass tubs were prepared for culture by sealing their inside surfaces with polyurethane. A 5/8" PVC pipe studded with 5 aquarium aerators is wedged lengthwise into the bottom of each tub. Filtered building air is used to aerate the cultures (Whatman 12-20 grade filter tubes). Each tub is covered by a sheet of plexiglass elevated slightly above the top of the tub by rubber stoppers at each corner. Banks of four 4-foot Duro-Test Vita Lites (40 watts) are suspended above each tub. The incident light passing through the plexiglass and reaching the surface of the culture is 80-100 $\mu E/m2/s$. The medium used is BG-11. The medium is prepared by first filling the tubs with deionized water that has been filter-sterilized through a 0.22 micron Millipack 200 filter unit. Nutrient salts are dissolved separately in 1-or 2-liter flasks, autoclaved, and then added to the water-filled tubs. Aeration is used to mix the contents, inoculum (12 liters) is added, allowed to mix, and then the air and light are removed overnight. More inoculum may be added later, depending on the growth of the culture. The total contents of the tubs are harvested every three weeks. The 200 liter contents of each tub are reduced during harvesting to about 2.5 liters with a Pelicon Millipore cell concentrator system. Concentration is done in about four

hours, with cell recovery over 95%. If the cells are healthy and the culture is not lysing, the toxin is retained within the cells. The concentrated cells are freeze-dried and stored in a -18°C freezer until they are extracted.

A. flos-aquae 525-17 is also semi-continuously cultured in (5) 180-liter cylinders at 22-25°C. These cylinders have 5/8" PVC pipes with air holes drilled into the bottom 6 inches extending the length of the cylinders, and custom-built plexiglass lids through which air is added to the culture and exhaust is vented. Banks of two 4-foot Duro-Test Vita-Lites (40 watt) are suspended beside the cylinders. The incident light passing through the fiberglass and reaching the surface of the cultures is 80-100 μE/m2/s. Filtered room air is used to aerate the cultures (Whatman 12-20 grade filter tubes and Millipore-FG 0.2 micron filter units). The medium used is BG-11. Medium and nutrient salts are added to the cylinders in the same way that they are added to the fiberglass tubs. Inoculum (24 liters) is added and allowed to mix. Aluminum foil is attached to the backs of the cylinders in front of the light banks to regulate the incident light, especially during the first 1 or 2 weeks of growth. More inoculum may be added 2 or 3 days later, depending on the growth of the culture. These cylinders are harvested once per week by removing 24 liters via a stopcock at the base of the cylinder, and replacement of the volume cylinder. The 24 liter sample is reduced to approximately 2.5 liters with the Pellicon cell concentrator. The concentrated cells are freeze-dried and stored.

Aphanizomenon flos-aquae strain NH-5-a is semi-continuously cultured in (9) 20 liter Bellco spinner flasks to provide cells for extraction of saxitoxin and neosaxitoxin. These cultures are kept at 22-25°C. The flasks are illuminated with Vita-Lite fluorescent bulbs and are aerated with filtered room air passed through glass aerators. The aerators are inserted into Consolidated Plastics bulkhead unions on the left cap of the spinner flasks. The right cap holds a glass elbow vent tube. The culture is sampled and medium (BG-11) aseptically replaced through this tube. Various harvest volumes and lengths of time between harvests were compared to find the most productive combination without depressing the growth of the culture. Presently, 8-9 liters are taken once per week from each flask. Initial set-up of the flasks involves autoclaving about 12 liters of BG-11 medium in each flask and inoculating it with 4 liters of log phase culture. Sampling of the cultures and replacement of volume with sterile media is done by syphoning out the algae and draining in the sterile media from an elevated 9 liter bottle through the glass elbow tube. Cells harvested from all flasks on a given date are combined, concentrated, and freeze-dried. Freeze-dried material is stored at -18°C until it is extracted.

Microcystis aeruginosa strain M-228 is grown to provide material for extraction of microcystin-YR. It is grown in one 180-liter cylinder which is managed as the other 180-liter cylinders, with BG-11 used as the medium. It is also grown in

(1) 20-liter Bellco spinner flask, managed as the other 20-liter spinner flasks.

Microcystis aeruginosa strain UV-027 is semi-continuously cultured to provide material for extraction of Microcystin-RR using (9) 20-liter Bellco spinner flasks. Again, these are managed as the other 20-liter spinner flasks, with BG-11 used as the medium.

Nodularia spumigena strain L-575 is batch cultured in (8) 12-liter bottles to provide cells for extraction of the cyclic pentapeptide nodularin. The cultures are kept at 22-25°C. They are illuminated with Vita-Lite fluorescent bulbs and aerated with filtered air passed through glass aerators. Presently, the total volumes of the bottles are harvested every 3-4 weeks. These volumes are reduced to 2.5 liters, freeze-dried, and stored at -18°C.

Microcystis aeruginosa strain PCC-7820 (nonaxenic) is cultured to provide material for extraction of Microcystin-LR. It is semi-continuously cultured in (4) 20-liter Bellco spinner flasks. Again, these are managed as the other 20-liter flasks, with BG-11 as the medium.

PCC-7820 (nonaxenic) is also batch cultured in one 90-liter plexiglass cylinder kept at 22-25°C. A 1/8" PVC pipe with air holes drilled into the bottom 6" extends the length of the cylinder. Filtered room air is used to aerate the culture. Banks of 40 watt Vita-Lites provide incident light. The medium used is BG-11. Sixty-eight liters of sterile BG-11 is added to the cylinder by pouring it into the top. This is allowed to mix by aeration. Inoculum (12 liters) is added and allowed to mix. Additional inoculum may be added later depending on the growth of the culture. The total volume is harvested every 4-5 weeks. The approximately 80 liters are reduced to about 2.5 liters, freeze-dried, and stored.

Anabaena flos-aquae strains IG-20 and 44-1-s-30 are grown in (3) and (2) 20-liter Bellco spinner flasks, respectively, to provide material for the extraction of anatoxin-a(s) and Anatoxin-a, respectively. Both are managed as the other 20-liter spinner flasks, with ASM-1 used as the medium.

Anabaena flos-aquae strain IC-1 and Anabaena sp. VS-1 are batch cultured in (2) 12-liter and (4) 12-liter bottles, respectively, with ASM-1-(minus NO3) and Z-8 used as the media, also respectively. These cultures are managed much like L-575 and the total volumes of the bottles are harvested every 3-4 weeks. Strain IC-1 produces anatoxin-a which strain VS-1 (Vermont-Star Lake) produces an unknown cytotoxin.

A new culture of Microcystis aeruginosa PCC-7820 (axenic) is semi-continuously cultured in (2) 20-liter Bellco spinner flasks, and since it is axenic it will eventually replace nonaxenic PCC-7820 in all cultures.

Table 4 and Table 5 summarize current culture volumes, LD50's, and yields of cells.

Table 4. Summary of Volumes Currently Being Used for Cultures of Cyanobacteria

Genus Species	Strain	Source of Culture	Current Culture Volume(L)	Average Yield of cells (g/L)	Toxin	LD_{50} i.p. mouse lyophilized cells	Culture medium
A. flos-aquae	NRC-525-17-b-1-e	N.R.C.	1300	0.0961	Anatoxin-a(s)	<190	BG-11
Aph. flos-aquae	NH-5-a	New Hampshire	210	0.1924	Aphantoxin (Neurotoxin)	<110	BG-11
M. aeruginosa	M-228	Japan	200	0.2523	Microcystin	<157	BG-11
M. aeruginosa	UV-027	Hartbeesport Dam, R.S.A.	180	0.2642	Microcystin-RR	<120	BG-11
N. spumigena	L-575	New Zealand	160	0.2641	Hepatotoxin	<176	BG-11+NaCl
M. aeruginosa	PCC-7820 (7820-old)	P.C.C.	140	0.1429	Microcystin-LR	<50	BG-11
A. flos-aquae	IG-20	Illinois	60	0.1530	Anatoxin-a(s)	<215	ASM-1
A. flos-aquae	44-1-s-30	44-1-s (N.R.C.)	40	0.3519	Anatoxin-a	nontoxic	ASM-1
Anabaena sp.	VS-1	Vermont	40	0.3170	Cytotoxin	not tested	Z-8
A. flos-aquae	IC-1	Idaho	20	0.1849	Neurotoxin	<100	ASM-1-0 (NO_3)
M. aeruginosa	PCC-7820 (7820-Pasteur)	P.C.C.	40	grown 10/89 to present	Microcystin-LR	not tested	BG-11

P.C.C. = Pasteur Culture Collection, Paris, France
Illinois = field isolate, Griggsville, Illinois
Japan = field isolate
N.R.C. = National Research Council, Ottawa, Canada
New Zealand = field isolate
New Hampshire = field isolate
Hartbeesport Dam, Republic of South Africa = field isolate
Vermont = field isolate

Table 5. Quarterly Summary of Dry Weight Cell Yields
(October 1988 - September 1989)
(grams/Liter)

Culture	1st Quarter Oct 88 - Dec 88	2nd Quarter Jan 89 - Mar 89	3rd Quarter Apr 89 - Jun 89	4th Quarter Jul 89 - Sep 89	TOTALS
525-17-b-1-e	171.96/2404	127.296/1288	148.256/2423	103.164/1620	550.676/7735
NH-5-a	25.336/104	26.905/156	32.561/172	59.885/441	144.687/873
M-228	39.559/324	50.109/340	30.049/97	44.564/300	164.281/1061
UV-027	---	8.26/32	61.097/306	94.708/675	164.065/1013
L-575	111.937/420	57.329/192	76.55/274	41.394/180	287.210/1066
7820-nonaxenic	181.624/1445	199.445/1287	164.766/1166	64.688/410	610.523/4308
IG-20	18.468/87	11.858/68	18.706/126	21.933/168	70.965/449
44-1-s-30	---	---	1.319/3	19.913/121	21.232/124
VS-1	---	---	3.938/16	10.677/30	14.615/46
IC-1	---	---	1.647/8	4.260/26	5.907/34
7820-axenic	---	---	---	---	grown 10/89 to present time

2. Timeline for Culturing and Harvesting Toxic Cyanobacteria.

a. Timeline involved in growing batch cultures of Anabaena flos-aquae NRC 525-17 in 200-liter tubs:

About 15 days	1)	Growing 25 ml Delong flasks
About 15 days	2)	Growing 1-L Delong flasks
About 30 days	3)	Growing 4-L Delong flasks
About 45 days	4)	Growing 12-L bottles
About 2 hrs/3 wks	5)	Cleaning, sterilizing two 200-L tubs
About 2 hrs/3 wks	6)	Filter-sterilizing water for tub cultures
About 2 hrs/3 wks	7)	Preparing tub media
Ten to twenty min	8)	Inoculating the tubs
About 21 days	9)	Allowing the cultures to grow
About 4 hours	10)	Harvesting the tub cultures
About 72 hours	11)	Freeze-drying the harvested cells
About 1 hr/3 wks	12)	Bottling, storing, & logging the dried cells

b. Timeline involved in growing semi-batch cultures of Anabaena flos-aquae NRC-525-17 in 180-liter cylinders:

About 15 days	1)	Growing 25 ml Delong flasks
About 15 days	2)	Growing 1-L Delong flasks
About 30 days	3)	Growing 4-L Delong flasks
About 45 days	4)	Growing 12-L bottles
About 2 hrs/2 mos	5)	Cleaning, sterilizing each 180-L cylinder
About 2 hrs/2 mos	6)	Filter-sterilizing water for the cylinders
About 2 hrs/2 mos	7)	Preparing media for the cylinders
Ten to twenty min	8)	Inoculating the cylinders
About 30 days	9)	Allowing the cultures to grow
About 3 hours	10)	Harvesting 24 liters from each cylinder
About 72 hours	11)	Freeze-drying the harvested cells
About 1 hr/week	12)	Bottling, storing, & logging the dried cells

c. Timeline for growing Microcystin aeruginosa PCC 7820 (nonaxenic) in semi-batch (20-liter) and batch (90-liter) cultures; also, timeline for growing PCC 7820 (axenic) in semi-batch (20-liter) culture:

About 45 days	1)	Growing inoculum for 20 L & 90 L cultures
About 2-3 hrs/vessel	2)	Sterilizing & setting up the culture vessels
About 6 hrs/wk	3)	Preparing media for the 20 L flasks
About 21 days	4)	Allowing 20 L cultures to grow
About 4-5 wks	5)	Allowing 90 L cylinder to grow
About 3 hrs/wk	6)	Harvesting 8 L from each flask
About 3 hrs/4-5 wks	7)	Harvesting entire 90 L cylinder
About 2 hrs/wk	8)	Replacing media in flasks
10-15 minutes	9)	Replacing media in the cylinder
About 72 hrs	10)	Freeze-drying the cells
About 1 hr/wk	13)	Bottling, logging, and storing the dried cells

d. Timeline for growing semi-batch cultures of Microcystin aeruginosa strain M-228 in 20-liter flasks and in 180-liter cylinders

-- very similar to that of PCC 7820 in 20-liter flasks and of 525-17-b-1-e in 180-liter cylinders.

e. Timeline for growing batch cultures of Nodularia spumigena strain L-575, Anabaena circinalis strain IC-1, and Anabaena sp. VS-1:

About 45 days	4)	Growing inoculum for & setting up 12-L bottles
About 2 hours/strain	5)	Harvesting total volume of bottles; (this is done on a staggered schedule)
About 72 hours	6)	Freeze-drying the harvested cells
About 1 hr/3-4 wks	7)	Bottling, logging, & storing the dried cells

f. Timeline for growing semi-batch cultures of Anabaena flos-aquae strains 44-1-s-30 and IG-20 in 20-liter flasks:

-- very similar to that of PCC 7820 in 20-liter flasks with harvest occurring every two weeks.

g. Timeline for growing semi-batch cultures of Aphanizomenon flos-aquae strain NH-5-a and Microcystis aeruginosa strain UV-027 in 20-liter flasks:

-- very similar to that of PCC 7820 in 20-liter flasks.

3. Recloning of A. flos-aquae NRc-44-1 -- Producer of Anatoxin A.

Single filament isolates from A. flos-aquae strain 44-1-s were done when it was found that the LD_{50} had risen greater than 250 and in some case was non-toxic. Isolates (usually varying in ability to produce toxin) were made in two ways:

1) Isolates were made by pipetting a few filaments from the culture onto a clean microscopic slide. A drop of sterile media was added to the colonies; gentle blowing on the drop through a Pasteur pipet dispersed the colonies. A desired filament was located in the drop using an inverted compound microscope. The filament was then drawn into a Pasteur pipet by capillary action (the pipet tip was tapered on a flame to allow more accuracy in selecting a single filament). The single filament was transferred to a second drop of sterile media, gentle blowing was used to separate it from any other filaments or debris, and the filament was finally transferred to a culture tube containing 1-2 ml of sterile media. Each isolate was coded with the original culture name (i.e. 44-1-s) and a number designating its position in the total number of isolates made. Surviving isolates are currently being cultured and tested for toxicity.

2) Isolates were made by inoculating sterile agar (ASM-1) in a petri-dish with one drop of culture, and allowing the culture to grow. When the cyanobacterial cultures appeared on the agar, each individual colony was transferred to a culture tube containing 1-2 ml of sterile media. Each isolate was coded as in procedure #1. Surviving isolates are currently being cultured and tested for toxicity.

3) Of the isolates tested thus far for toxicity, two (44-1-s-27 and 44-1-s-30) tested positive and, one (44-1-s-30) is currently semi-continuously cultured in two 20-liter Bellco spinner flasks. However, when last tested for toxicity, 44-1-s-30 tested non-toxic; therefore, 44-1-s-27 and the remaining clones will be grown to volumes sufficient for testing.

4. Field Sample Testing and Algal Strain Isolation. Maintenance and Preservation of Field and Culture Strains of Cyanobacteria.

a. Field sample testing and algal strain isolation.

The laboratory received incoming samples of potentially toxic algae from various sources, including public water systems, governmental health agencies, and other university-associated and independent parties. Some of these field collections were samples of algal blooms, with a high density of biomass and cells suspected of being toxigenic; other field collections were less concentrated samples of water from varous survey points to monitor the presence of potentially toxic algae.

Once collected, the samples were sent via overnight delivery to the laboratory. When possible, samples were collected using a standard sample kit prepared by this laboratory and mailed prior to collection to the corresponding agency. These kits included the following: 1) two 500 mL plastic screw-cap bottles to collect adequate sample for lyophilization and toxicity testing; 2) two 25 mL screw-cap culture tubes with 10 mL BG-11 culture medium to enhance survival of algae present; 3) two 25 mL screw-cap culture tubes with 10 mL of Lugol's preservative to preserve samples for microscopic examination and identification in the event the living material is altered; 4) two empty 25 mL screw-cap culture tubes to collect sample for strain isolation; and 5) Blue Ice to keep the sample cool during return shipment.

Upon receipt, these kits were immediately processed. The contents of all containers were microscopically examined to confirm the initial report and to note the differences, if any, among the living, preserved, and media-enriched samples. The large living samples were lyophilized, and the media-enriched tubes were placed in an incubator. The small living samples were refrigerated until the algal strain isolations were performed, within 72 hours. The preserved samples were microscopically examined for identification of the genera, and if possible, the species present.

Isolation of the likely toxigenic algal strains in the samples was initiated once the toxicity of the parent material was confirmed by mouse intraperitoneal bioassay. These isolations were performed utilizing two methods: core isolates and drop isolates. Isolation by cores involved the following steps: 1) dilution of the field sample, usually 1:10 or 1:100; 2) inoculation of 1.5% soft agar plates (mixed with BG-11, ASM-1, or Z-8 nutrient media before cooling) with 0.5 mL of sample dilution; 3) sealing of plates and storage in an incubator for 24-72 hours; 4) identification and marking of individual filaments or colonies on the plates by use of an inverted microscope; 5) isolation of the core of soft agar containing the colony or filament by suction-drawing into a fine-tipped sterile pipette; 6) inoculation of the core into a small culture tube with 2 mL of sterile BG-11, ASM-1, or Z-8 media (see Table 6).

Isolation by drops involved the following steps: 1) pipetting of a drop of dilute sample onto a sterile microscope slide; 2) placing of two separate nonconfluent drops of sterile media upon the same slide; 3) drawing up of a single colony or filament from the dilution sample into a flame-tapered fine tipped pipette; 4) inoculation of the colony or filament into a drop of media; 5) successive transfer of the single filament or colony to the third drop of media; and 6) inoculation of the single filament or colony from the third drop into a small culture tube with 2 mL of either BG-11, ASM-1, or Z-8 media.

Once the single filament or colony tubes were inoculated by either core or drop isolation, they were placed in an incubator under 40-60 microE/m2/s at 24°C. The tubes were examined at regular intervals for macroscopic evidence of growth, which if evidenced, was followed by microscopic examination, to confirm that the alga

Table 6. Culture Media for Growth of Toxic Cyanobacteria

Nutrient	ASM-1 (mg/L)	BG-11 (mg/L)	BG-11 (L-575) (mg/L)	Z-8 (mg/L)	Z-8 with salt (mg/L)	Z-8 without nitrogen (mg/L)
$NaNO_3$	170.00	1500.00	750.00	467.00	467.00	---
K_2HPO_4	17.40	40.00	40.00	31.00	31.00	31.00
Na_2HPO_4	14.20	---	---	---	---	---
$MgCl_2$	19.02	---	---	---	---	---
$MgSO_4 \cdot 7H_2O$	49.32	75.00	75.00	25.00	3775.00	25.00
$CaCl_2 \cdot 2H_2O$	29.40	36.00	36.00	---	37.00	
Citric Acid	---	6.00	6.00	---	---	---
Na_2CO_3	---	20.00	20.00	21.00	21.00	21.00
Na_2EDTA	6.64	1.00	1.00	---	---	---
Ferric Ammonium Citrate	---	6.00	6.00	---	---	---
NaCl	---	---	7000.00	---	8750.00	---
$Ca(NO_3)_2 \cdot 4H_2O$	---	---	---	59.00	59.00	---
Fe - EDTA	---	---	---	0.344	0.344	0.344

ASM-1 minor elements: (mg/kg in culture medium) $FeCl_3$ - 0.65, H_3BO_3 - 2.47, $MnCl_2 \cdot 4H_2O$ - 0.87, $ZnCl_2$ - 0.44; $CoCl_2 \cdot 6H_2O$ - 0.01, $CuCl_2 \cdot 2H_2O$ - 0.0001 (In our laboratory, Tris is added at the level of 26.90 mg/12 - this provides better buffering of the medium and it increases the length of storage for unused media). ASM-1 is adjusted to pH 8.5 with 0.5 NaOH before autoclaving.

BG-11 minor elements: (g/L) H_3BO_3 - 2.86, $MnCl_2 \cdot 4H_2O$ - 1.81, $ZnSo_4$ - 0.222, $Na_2MoO_4 \cdot 2H_2O$ - 0.39, $CuSo_4 \cdot 5H_2O$ - 0.079, $Co(NO_3)_2 \cdot 6H_2O$ - 0.049. Add 1 ml/L into the culture medium. After autoclaving and cooling, pH of the medium is about 7.1.

Z-8 minor elements: (g/L) $Na_2WO_4 \cdot 2H_2O$ - 0.33, $(NH_4)_6\ Mo_7O_{24} \cdot 2H_2O$ - 0.88, KBr - 1.20, KI - 0.83, $ZnSO_4 \cdot 7H_2O$ - 2.87, Cd $(NO_3)_2 \cdot 4H_2O$ - 1.55, Co $(NO_3)_2 \cdot 6H_2O$ - 1.46, $CuSO_4 \cdot 5H_2O$ - 1.25, $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ - 1.98, $Cr(NO_3)_3 \cdot 9H_2O$ - 0.41, V_2O_5 - 0.089, $Alz(SO_4)_3K_2SO_4 \cdot 24H_2O$ - 4.74, H_3BO_3 - 3.10, $MnSO_4 \cdot 4H_2O$ - 2.23

growing was the isolate of interest and not a contaminant. If the strain was growing free of contamination, it was given a name based upon this laboratory's nomenclature and then successively cultured. Once a sufficient quantity of cells is obtained, lyophilization of the cells was performed so that a mouse bioassay could confirm the toxicity or nontoxicity of the isolated strain.

Table 7 summarizes the field sample data for the samples received: collection source, strain designation, date of collection, reason for collection, LD-50, toxicity by mouse bioassay, genera described by microscopic examination, number of core isolates and number of drop isolates.

b. Maintenance and preservation of field and culture strains of cyanobacteria.

A total of 68 strains of cyanobacteria were maintained throughout the year by transfer of the unialgal culture into fresh media at four week intervals. These cultures were maintained in duplicate in 25 mL screw-cap culture tubes incubated at 30 microE/m2/s at 24°C. The strains include various toxic and non-toxic representatives of the following genera:

Anabaena	30 strains
Microcystis	18 strains
Oscillatoria	8 strains
Anacystis	3 strains
Pseudanabaena	3 strains
Lyngbya	3 strains
Calothrix	1 strain
Plectonema	1 strain
Schizothrix	1 strain
Synechocystis	1 strain
Selenastrum	1 strain
Gleocapsa	1 strain
Gloeotrichia	1 strain
Scytonema	1 strain

Each strain was maintained in the medium or media among BG-11, ASM-1, or Z-8 in which growth was optimal.

TABLE 7. Field Samples Received: October 1988 → October 1989

Collection Source	Strain Designation	Collection Date	Toxicity Mouse LD_{50}	Algal genera present	Number of core isolates	Number of drop isolates
Crooked Lake, Indiana	---	4-28-89	no bioassay	Oscillatoria	0	0
Lake Washington, Indiana	---	4-28-89	no bioassay	Oscillatoria agardhii	0	0
Devil's Lake, North Dakota	NDD	6-9-89	NT @ 1000 mg/kg	Synechocystis, Microcystis	0	0
		7-21-89	H @ 375 mg/kg	Microcystis aeruginosa Microcystis weisenbergii	30	0
		7-31-89	NT @ 1000 mg/kg	Synechocystis	0	0
		8-8-89	H @ 1000 mg/kg	Microcystis aeruginosa	10	0
		8-11-89	H @ 750 mg/kg	Microcystis aeruginosa	24	0
		8-16-89	NT @ 1500 mg/kg	Synechocystis, Microcystis aeruginosa	0	0
		8-31-89	H @ 500 mg/kg H @ 750 mg/kg	Microcystis aeruginosa Microcystin weisenbergii	20 10	0 0
		9-14-89	NT @ 1500 mg/kg	Lyngbya, Oscillatoria, Microcystis aeruginosa	0	0
		9-25-89	NT @ 2000 mg/kg	Microcystis aeruginosa	16	0
Fort Peck, Montana	MTF	8-11-89	H @ 75 mg/kg	Anabaena	30	10
Eagle River Flats, Alaska	---	10-12-89	NT @ 2000 mg/kg	Oscillatoria	0	0

NT = nontoxic; H = hepatotoxic

Table 8 summarizes the data for all 68 strains of cyanobacteria in the live algae collection at Wright State University.

The headings of the table are defined below:

THE LIVE ALGAE COLLECTION AT WRIGHT STATE UNIVERSITY

GENUS, SPECIES, & STRAIN:
The taxonomic names of species given, if known. Some strains are identified by names given to them by the collectors from whom they were received, especially if these strains have been described and catalogued by these names. Other strains, isolated by this lab from field material or mixed-strain samples received, are given a WSU strain number, with a two-letter prefix and a number indicating the number of isolates from that site. For instance, IC-1 indicates the first isolate from Cave Lake, Idaho.

SOURCE:
Indicates by letter the type of toxicity, if any, exhibited by the strain upon test organisms:

H: hepatotoxicity
N: neurotoxicity
D: dermotoxicity
C: cytotoxicity
NT: non-toxic

The relative toxicities for "H" and "N" are given in LD_{50} values for milligram (mg) freeze-dried cells of algal strain kilogram (kg) of test animal body weight, when a suspension of freeze-dried cells is administered intraperitoneally (IP) into a test mouse. The dates of testing follow.

MEDIA:
Indicates the growth medium or media used to support the living cultures.

COMMENTS:
May indicate the person or institution who collected the material, isolated the strain, provided the culture, or other salient information.

LIVE ALGAE COLLECTION: CARE INSTRUCTIONS

The eight racks, #1-8, are maintained in duplicate for a total of 16 racks. The eight racks of 12 ml culture tubes contain the various strains, three tubes of each strain per rack. One tube is typically older in age, is situated in the left-hand row of the rack, and provides the inoculum for the two "new" tubes at each transfer. These two "new" tubes are located in the two right-hand rows of the rack. At each transfer, one "old" tube inoculates two freshly inoculated tubes that become the "new" tubes, and one of the two formerly "new" tubes becomes an "old" tube, stored in the left-hand column. If all the tubes are healthy, the one extra

formerly "new" tube, now "old", may be discarded. If there is any questions about the fitness of any of the tubes, the extra tube should be retained. If, at any time, the "old" tube is not healthy and does not provide good inoculum, the extra tube may be used as inoculum.

Some strains do not grow well in culture tubes and thus are maintained additionally in 25 ml DeLong flasks. They are kept on a tray, maintained in quadruplicate and transferred weekly.

The strains in the culture tube racks should typically be transferred monthly. If two racks, in duplicate, are transferred weekly, a convenient schedule for the eight racks can be arranged. It is often convenient to transfer the two duplicates of each rack number simultaneously so that the relative health of all six tubes can be compared. If necessary, inocula from tubes in one rack may be used to prepare tubes for the other. The healthiest, cleanest tubes should be used as inoculum for the new tubes. This often requires microscopic examination, which should be done regularly, at least at every second transfer. A healthy green color may only indicate that a contaminant is growing well.

Some strains have a history of easily contaminating other strains. These "weedy" strains are maintained separately in rack #8, at a location where they are not physically close to the other strains. The other seven racks, in duplicate, are maintained at two separate locations, racks #1-7 at one site, the duplicate racks #1-7 at another. This ensures that mechanical or operational failure of one incubator's light, temperature, or electrical controls does not adversely effect the entire culture collection.

Density of inoculum may vary with the strain, but an inoculation volume of about 20% is probably optimal. Some strains, like Oscillatoria, the various Lyngbya species, and Anacystis, may require much smaller volumes; other times, some weak strains, as Anabaena flos-aquae 1444, may require use of 30-40% if the abundance of filaments in the parent culture is very low.

The live algae are maintained at 24°C with a light flux density of about 30 micromoles per meter squared per second. One set of tubes (racks #1-7) has been maintained on a 12:12 dark:light cycle every 24 hours. This may be a beneficial practice for all collections as it may prevent photorespiration, and possible dark cycle heterotrophy of some cyanobacterial strains may reduce available substrate for bacteria. All tubes should be shaken individually and fairly vigorously about once a week if possible to aerate and suspend non-vacuolated strains.

TABLE 8. Live Algae Collection at Wright State University

Genus/Species	Strain	Isol. Date	Source	Toxicity	Media	Comments
Selanastrum capricornutum	---	---	---	---	BG-11	From EPA
Anacystis nidulans	---	---	---	NT	BG-11	----
Anacystis sp.	IU-549	---	---	NT	BG-11	----
Anacystis nidulans	IU-550	---	---	NT	BG-11	----
Synechocystis cedrorum	---	---	---	NT	BG-11	From Carolina Bio. Supply via W. Demott
Gleocapsa sp.	---	---	---	---	BG-11	----
Gleocapsa sp.	---	---	---	---	BG-11	----
Microcystis sp.	(X-MA-81)	1986?	MA-81	---	BG-11	Found as contaminant
Microcystis sp.	O-1	1981	Lk. Erie,OH	H@300	BG-11	----
Microcystis sp.	OK-12	1985	Norman,OK	---	BG-11	----
Microcystis sp.	OK-16	1985	Norman,OK	---	BG-11	----
Microcystis aeruginosa	C-5	----	Wisconsin	NT	BG-11	From D. Parker
Microcystis aeruginosa	C3-40	----	Wisconsin	NT	BG-11	From D. Parker
Microcystis aeruginosa	A-272	1975	Alberta	H@60,1976	BG-11	See Special Care
Microcystis aeruginosa	A-207	1975	Alberta	H@60,1976	BG-11	See Special Care
Microcystis aeruginosa	UTEX-2061	----	Univ. of Texas	NT	BG-11	----
Microcystis aeruginosa	UTEX-2063	----	Univ. of Texas	---	BG-11	----
Microcystis aeruginosa	7820-Pasteur	1989	Scotland	---	BG-11	From Pasteur Ins. via Rosie Rippka, 1989
Microcystis aeruginosa	7820-Old	1976	Scotland	H@50	BG-11	Given "old" designation to distinguish from new 7820-Pasteur (axenic)
Microcystis aeruginosa	M-228	----	Japan	---	BG-11	----
Microcystis aeruginosa	UV-006	1978	Hartbeesport,RSA	H@200	BG-11	From J.N. Eloff
Microcystis aeruginosa	UV-027	----	Hartbeesport,RSA	H	BG-11	From J.N. Eloff
Microcystis aeruginosa	NRC-1 SS-17 CA	1958	Ontario	H@50	BG-11	----
Microcystis aeruginosa	SS-17 Norway	1958	Ontario	NT	BG-11	Norway nontoxic
Microcystis aeruginosa	SS-17	1958	Ontario	H@100	BG-11	Returned 10-84
Microcystis sp.	(X-M-4)	1986?	M-4	---	BG-11	Contaminant in M-4
Microcystis aeruginosa	M-53	1984	Montana	---	BG-11	----
Oscillatoria sp.	----	----	----	---	ASM-1	From Carolina Bio. Supply via W. Demott
Oscillatoria agardhii	Lk. Washington	----	Lk. Washington,WA	---	ASM-1	From W. Demott
Oscillatoria sp.	Crooked Lk.,SP2	----	Crooked Lk.,IN	---	ASM-1	From W. Demott

Genus/Species	Strain	Isol. Date	Source	Toxicity	Media	Comments
Oscillatoria agardhii	CYA-18	1971	Norway	H@100 D	ASM-1	From L. Gjersjoen
Oscillatoria agardhii	CYA-29	1968	Norway	H@100 D	ASM-1	From L. Gjersjoen
Oscillatoria agardhii	CYA-29-h	1986	CYA-29	---	ASM-1	See Special Care
Oscillatoria sp.	Lk. Mead A-1	----	----	---	ASM-1	See Special Care
Oscillatoria sp.	Finland B-1	----	----	---	ASM-1	See Special Care
Lyngbya borgetti	----	----	----	NT	BG-11	----
Lyngbya borgetti	IU-622	----	----	---	BG-11	----
Lyngbya versicolor	----	----	----	NT	BG-11	----
Gloeotrichia	MT(non unialgal)	----	Montana	---	ASM-1; ASM-1-0(NO3)	Contaminated with Oscillatoria; See Special Care
Calothrix	---	----	----	NT	BG-11	Morphology lost
Scytonema pseudohofmanii	---	----	----	---	Z-8	May produce cyanobacterin
Scytonema pseudohofmanii	---	----	----	---	ASM-1	May produce cyanobacterin
Plectonema boryanum	---	----	----	NT	BG-11	----
Pseudabaena brunea	---	----	Alberta	NT	BG-11	From Paul Gorham
Pseudabaena catenata	CCAP 1464-1	----	Alberta	NT	BG-11	From Paul Gorham
Pseudabaena catenata	----	----	Alberta	NT	BG-11	From Paul Gorham
Aphanizomenon flos-aquae	NH-5-a	1980	New Hampshire	N@5	BG-11	----
Aphanizomenon flos-aquae	NH-5-a-17	1985	NH-5-a	N@20	BG-11	----
Aphanizomenon flos-aquae	NH-5-a-36	1985	NH-5-a	N@25	BG-11	----
Anabaena sp.	VS-1	----	Star Lk.,VT	C	Z-8	May be A. affinis or A. levanderi
Anabaena sp.	VS-1	----	Star Lk.,VT	C	ASM-1-1/5 (NO3)	May be A. affinis or A. levanderi
Anabaena sp.	VS-1	----	Star Lk.,VT	C	ASM-1-0 (NO3)	May be A. affinis or A. levanderi
Anabaena sp.	VS-1-a	1989	VS-1	---	Z-8	----
Anabaena sp.	VS-1-b	1989	VS-1	---	Z-8	----
Anabaena sp.	VS-1-c	1989	VS-1	---	Z-8	----
Anabaena sp.	VS-1-d	1989	VS-1	---	Z-8	----
Anabaena sp.	VS-1-e	1989	VS-1	---	Z-8	----
Anabaena sp.	VS-1-f	1989	VS-1	---	Z-8	----

Genus/Species	Strain	Isol. Date	Source	Toxicity	Media	Comments
Anabaena sp.	VS-1-g	1989	VS-1	---	Z-8	----
Anabaena sp.	VS-1-h	1989	VS-1	---	Z-8	----
Anabaena flos-aquae	IG-20	1986	Griggsville,IL	N	ASM-1	----
Anabaena flos-aquae	IG-20	1986	Griggsville,IL	N	ASM-1-1/5(NO3)	----
Anabaena sp. (Jim's room)	OF-1	1979	Fairborn,OH	NT	BG-11	Wall of house
Anabaena flos-aquae	M-57	1984	Montana	---	BG-11	See Special Care
Anabaena flos-aquae	1444	----	----	---	BG-11	Univ. of Texas
Anabaena flos-aquae	S-25	1975	Saskatchewan	N@30	BG-11	----
Anabaena flos-aquae	S-23-g	1975	S-23	N@30	BG-11	S-23 from Sask., 1975; discarded 1975
Anabaena flos-aquae	S-23-g-1	----	S-23-g	N,H@100	BG-11	Toxin variable
Anabaena flos-aquae	S-23-g-1-c	----	S-23-g-1	H@100	BG-11	Exhibits no neurotox.; See Special Care
Anabaena circinalis	IC-1	1988	Cave Lk.IN	N	ASM-1-0 (NO3)	From D. Stevens; See Special Care
Anabaena flos-aquae	445-1 PRG	1961	Saskatchewan	N@300	ASM-1	From Paul Gorham 445-L? or 445-1?
Anabaena flos-aquae	445-H	1961	Saskatchewan	N,NOW,NT	BG-11	----
Anabaena flos-aquae	525	1965	Saskatchewan	N@270,1975	BG-11	----
Anabaena flos-aquae	525-17-b-1-e	----	525-17-b-1	N@40	BG-11	No record of intermediate clones between 525 & 525-17-b-1-e
Anabaena flos-aquae	44-1	1961	Saskatchewan	N@50	BG-11	----
Anabaena flos-aquae	44-1 (nontoxic)	----	----	NT	BG-11	----
Anabaena flos-aquae	44-1-s	----	44-1	N,NOW,NT	BG-11	----
Anabaena flos-aquae	44-1-s-9	1985	44-1-s	N@50,1985	BG-11	----
Anabaena flos-aquae	44-1-s-27	1988	44-1-s	---	BG-11	----
Anabaena flos-aquae	44-1-s-29	1985	44-1-s	N@40,1985	BG-11	----
Anabaena flos-aquae	44-1-s-30	1988	44-1-s	---	BG-11	----
Anabaena flos-aquae	44-1-u	----	44-1	N,NOW,NT	BG-11	----

5. Isolation, purification, and shipment of deliverables

Throughout the course of this contract methods for culture of toxic cyanobacteria have been improved. This relates to the area of cell growth and harvest. No studies were done specifically to address specific grown conditions that might affect toxin production. Since adequate toxin production was always correlated by good growth (i.e., biomass), the goals of this contract were basically met with available culture facilities. It is, however, hoped that further studies will be directed toward the factors regulating toxin production.

Isolation and purification procedures for the various toxins represented the area of most intensive study on this project. Procedures were constantly modified in minor ways to improve yields and shorten extraction times. No major changes were made over the past year (see annual report for 11/1/87-10/31/88). Procedures used in our laboratory are outlined in last year's annual report and in recent papers by Harada et. al. (1988a,b; 1989).

During the time period for this present annual/final report the following amounts of deliverables were made to USAMRIID:

1) microcystin-LR: 119.6 mg
2) microcystin-YR: 1.7 mg
3) nodularin: 283.6 mg
4) anatoxin-a(s): 43.8 mg

Other shipments were made to laboratories either collaborating/contracted with USAMRIID or doing collaborative work with the PI's laboratory (Table 9).

Table 9. Schedule of deliverables supported on contract DAMD17-87-C-7019 and on a subcontract from contract DAMD17-85-C-5241 (Univ. of Illinois-V.R. Beasley), for the time period November 1, 1988-April 30, 1990.

Date Sent*	Description	Amount(mg)	Receiver	Comments
11/21/88	Microcystin-LR	26.60	V. Beasley	Univ. of Illinois
12/5/88	Microcystin-RR	.25	F. Chu	Univ. of Wisconsin
12/5/88	Microcystin-YR	.13	F. Chu	Univ. of Wisconsin
12/5/88	Microcystin-LA	.10	F. Chu	Univ. of Wisconsin
12/5/88	Ozonated MCYST-LR	.07	F. Chu	Univ. of Wisconsin
12/9/88	Microcystin-LR	2.00	T. Foxall	Univ. New Hampshire
12/15/88	Microcystin-LR	51.00	D.L. Bunner	USAMRIID
12/15/88	Anatoxin-a(s)	4.00	D.L. Bunner	USAMRIID
12/15/88	Microcystin-YR	1.70	D.L. Bunner	USAMRIID
12/15/88	Anatoxin-a(s)	1.00	W. Cook	Univ. of Illinois
12/26/88	Nodularin	3.40	H. Fujiki	Tokyo, Japan
1/9/89	Microcystin-LR	12.30	D. Morton	Frostburg State Univ.
2/1/89	Anatoxin-a(s)	4.00	D.L. Bunner	USAMRIID
2/1/89	Anatoxin-a(s)	4.00	S. Matsunaga	Univ. of Hawaii
3/31/89	Nodularin	19.90	D.L. Bunner	USAMRIID
3/31/89	Microcystin-LR	68.50	D.L. Bunner	USAMRIID
3/22/89	Anatoxin-a(s)	4.00	D.L. Bunner	USAMRIID
3/22/89	Anatoxin-a(s)	5.10	S. Matsunaga	Univ. of Hawaii
3/22/89	Anatoxin-a(s) (degradation product-nontoxic)	7.60	S. Matsunaga	Univ. of Hawaii
4/10/89	Microcystin-LR	15.00	D. Morton	Frostburg State Univ.
5/4/89	Nodularin	52.90	D.L. Bunner	USAMRIID
5/22/89	Anatoxin-a(s)	4.00	D.L. Bunner	USAMRIID
6/8/89	Nodularin	55.30	D.L. Bunner	USAMRIID
6/27/89	Anatoxin-a(s)	3.00	D.L. Bunner	USAMRIID
7/12/89	Nodularin	40.90	D.L. Bunner	USAMRIID
7/24/89	Anatoxin-a(s)	3.00	D.L. Bunner	USAMRIID
8/23/89	Nodularin	40.40	D.L. Bunner	USAMRIID
8/28/89	Anatoxin-a(s)	2.80	D.L. Bunner	USAMRIID
9/20/89	Anatoxin-a(s)	2.00	D. Franz	USAMRIID
9/20/89	Nodularin	44.00	D. Franz	USAMRIID
9/26/89	Microcystin-LR	8.50	M. Namikoshi	Univ. of Illinois
9/27/89	Microcystin-LR	4.20	R. Moore	Univ. of Hawaii
12/12/89	Nodularin	30.20	D. Franz	USAMRIID
3/26/90	Anatoxin-a(s)	17.00	D. Franz	USAMRIID

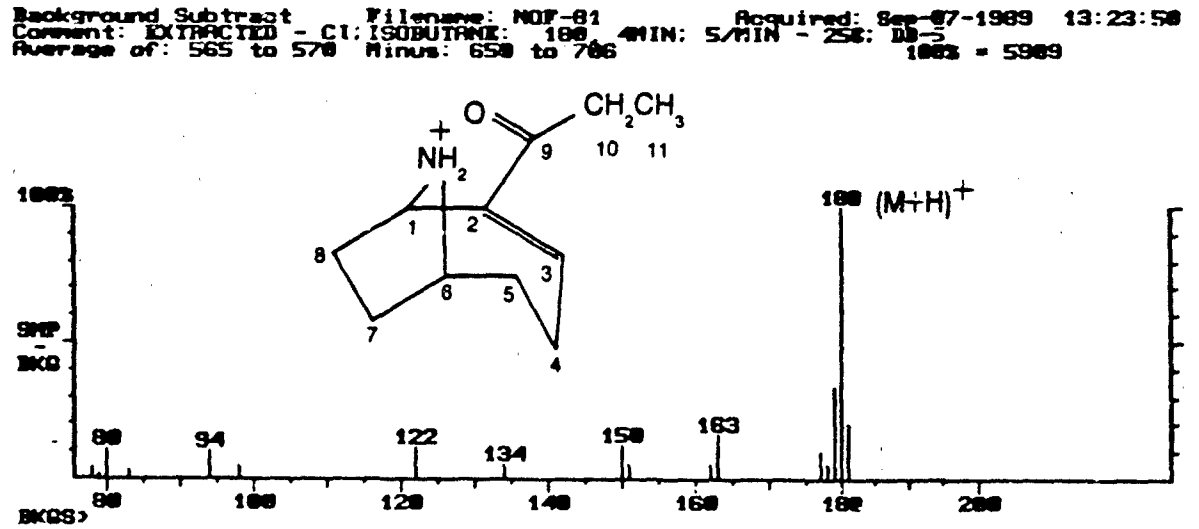
*All shipments to USAMRIID were Federal Express or UPS Express. Shipments were packed in DOT approved hazardous substances shipping containers - ALLPAC®, Pittsburgh, PA.

New toxic strains and toxins studied during this report period included homoanatoxin-a isolated from a Norwegian Oscillatoria strain (Fig. 5; O.M. Skulberg, Norwegian Institute Water Research, unpublished data) and new microcystin homologues isolated from a Finnish Nostoc strain (Fig. 4; Table 3, K. Sivonen, University of Helsinki, unpublished data).

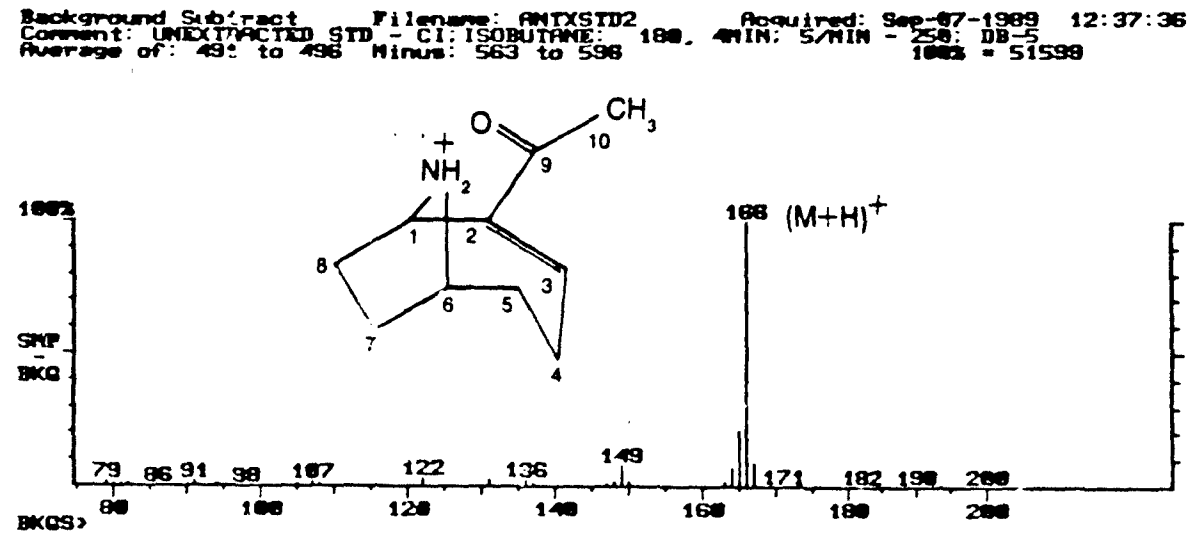
In addition the structure of anatoxin-a(s), a potent organophosphate irreversible anticholinesterase produced by certain strains of Anabaena flos-aquae was elucidated and published during this report period (Fig. 1; Appendix 1).

Fig. 5 Structure of homoanatoxin-a produced by the Norwegian Oscillatoria sp. strain number NOF-81 (A) and anatoxin-a produced by various Anabaena flos-aquae strains (B).

A.


Background Subtract Filename: NOF-81 Acquired: Sep-07-1989 13:23:50
Comment: EXTRACTED - CI: ISOBUTANE: 180, 4MIN: 5/MIN - 250: DB-5
Average of: 565 to 570 Minus: 650 to 706 100% = 5989
O
CH2CH3
NH2
9
10 11
1
2
3
4
5
6
7
8
100% (M+H)+
SMP
-
BKG
80
94
122
134
150
163
BKGS>

B.


Background Subtract Filename: ANTXSTD2 Acquired: Sep-07-1989 12:37:36
Comment: UNEXTRACTED STD - CI: ISOBUTANE: 180, 4MIN: 5/MIN - 250: DB-5
Average of: 491 to 496 Minus: 563 to 598 100% = 51598
O
CH3
NH2
9
10
1
2
3
4
5
6
7
8
100% (M+H)+
SMP
-
BKG
79
86
91
98
107
122
136
149
171
182
198
200
BKGS>

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C. PROJECT SUMMARY

Toxic waterblooms of freshwater cyanobacteria are unpredictable and intermittent in occurrence. They are most often found in temperate latitudes and occur in shallow inland reservoirs, lakes, ponds, rivers, and sloughs. Cases of blue-green algae toxicosis have been verified in every continent except Antarctica. They are particularly abundant and increasingly recognized in the inland water bodies of Central/Eastern Europe, Western Asia (Ukraine), Southeast Asia/India/Japan, Southern Africa, South America and North America. An increasing number of these cases involve human contact with toxic blue-green algae, although at this time no confirmed deaths due to the toxins have been reported. Toxin groups include alkaloids, peptides and contact poisons. The alkaloids currently include anatoxin-a (a depolarizing neuromuscular blocking agent), anatoxin-a(s) (an irreversible anticholinesterase), and aphantoxin-I and II (equivalent to neosaxitoxin and saxitoxin, the major paralytic shellfish toxins). Peptide toxins are a family of cyclic hepta- and pentapeptides with similar activity. They primarily act as hepatotoxins, causing hepatocyte disaggregation and death by hemorrhagic shock. The contact toxins are at present poorly understood but current information suggests they are not related to the other blue-green toxins. All of these toxins represent potential threat agents because they are: 1) water soluble and orally toxic; 2) accumulate in high concentrations (algal blooms) making them relatively easy to collect and process into highly concentrated crude toxin preparations.

This report represents work supported by USAMRDC during the period November 1, 1988 to April 30, 1990. The contract contributed to the establishment of a culture facility which supplied research level quantities of known freshwater blue-green toxins. Cyclic peptide toxins were used for basic investigations leading to an understanding of structure, function, and detection methods for these toxins. This contract supported the culture facility (which in turn, provided material for the inhouse projects at USAMRIID) and allowed further work on other freshwater blue-green algal toxins.

D. Papers Published in the Scientific Literature, and Presented at Scientific Meetings supported in part by Contract DAMD-17-87-C7019 (annual/final report year 1988-90).

Scientific Paper (P)/Poster (PO) Presentations
(Presenter is underlined)

PO Non-anticholinesterase effects of Anatoxin-a(s). Society of Toxicology, 1990 Annual Meeting. Miami, Florida. Feb. 1990. (E.G. Hyde and W.W. Carmichael). Abstract.

PO Uptake and intracellular localization of ^{3}H-Microcystin-LR in perfused liver and hepatocyte suspension. Soc. of Toxicologic Pathologists - VIII Int. Symp. Cincinnati, Ohio. May 21-25, 1989. (S.B. Hooser, M.S. Kuhlenschmidt, V.R. Beasley, W.W. Carmichael and W.M. Haschek).

PO Some structure function studies on Anatoxin-a(s). Gordon Research Conference on Mycotoxins and Phycotoxins. Plymouth, NY. June 26-30, 1989. (W.W. Carmichael and E. Hyde).

PO Toxicities and toxins of cyanobacteria waterblooms collected from inland waterbodies in central China. (Qing-Xue Zhang, W.W. Carmichael, M-J. Yu and S-H. Li). Gordon Research Conference on Mycotoxins and Phycotoxins. Plymouth, NH. June 26-30, 1989.

PO Toxicity of blue-green algae (cyanobacteria) waterblooms in central China. International Symposium of Natural Toxins. Nanning, Guangxi, P.R. China. May 22-25, 1989. (M-J. Yu, Q-X. Zhang, J-W. He, Z-R. He and W.W. Carmichael).

P Mechanisms and structure/activity relationships of cyanobacterial cyclic peptide hepatotoxins. Third International Symposium on Toxic Plants. Logan, Utah. July 23-29, 1989. (V.R. Beasley, W.W. Carmichael, C. Chen, A.M. Dahlem, W.M. Haschek, S.B. Hooser, M.S. Kuhlenschmidt, R.A. Lovell, M. Namikoshi, and K.L. Rinehart).

PO The role of α, β-unsaturated amino acids in the toxicity of microcystin-LR and nodularin, two hepatotoxins from cyanobacteria. Society of Toxicology - 1989 Annual Meeting. Atlanta, Georgia. March 1989. (A.M. Dahlem, V.R. Beasley, K-I Harada, K. Matsuura, M. Suzuki, C.A. Harvis, K.L. Rinehart and W.W. Carmichael).

Scientific Publications (I - invited, R - reviewed, Ref - Refereed)

Ref Hooser, S.B., V.R. Beasley, E.J. Basgall, W.W. Carmichael and W.M. Haschek. 1990. Ultrastructural changes induced by microcystin-LR in rats. Vet. Pathol. 27: 9-15.

IR Carmichael, W.W., N.A. Mahmood and E.G. Hyde. 1990. Natural toxins from cyanobacteria (blue-green algae). In S. Hall and G. Strichartz (eds.), Marine Toxins: Origin, Structure, and Molecular Pharmacology. ACS - Symposium Series #418, American Chemical Society, Washington, DC, pp. 87-106.

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Rev. 4-90

APPENDICES

APPENDIX I

Anatoxin-a(s), a Potent Anticholinesterase from *Anabaena flos-aquae*

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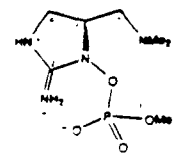
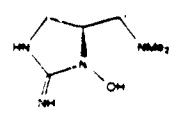
Received June 1, 1989

Anatoxin-a(s) is a neurotoxic alkaloid associated with the blue-green alga *Anabaena flos-aquae*.[1] It potent toxicity (LD_{50} 20–40 μg/kg mice) is attributed to exceptional anticholinesterase activity.[2] We report here the isolation of anatoxin-a(s) from a cultured strain NRC 525-17 and a field-collected bloom implicated in animal poisonings[3] and the determination of its structure as **1**.



1



2

Freeze-dried alga was extracted with 0.05 N AcOH/EtOH. The filtered extract was partitioned between water and CH_2Cl_2, the aqueous layer was washed with *n*-BuOH and evaporated in vacuo, and the residue was extracted successively with small portions of 0.05 N AcOH/MeOH and 0.05 N AcOH/EtOH to give a toxic concentrate. Gel filtration on Toyopearl HW40F (Supelco)[4] followed by HPLC on CN and ODS columns gave pure anatoxin-a(s) as a colorless solid in 0.05% yield. Toxin isolation was followed by assaying fractions for anticholinesterase activity.[5] Anatoxin-a(s) decomposed rapidly in basic solution but was

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(2) (a) Mahmood, N. A.; Carmichael, W. W. *Toxicon* **1987**, *25*, 1221. (b) Cook, W. O.; Beasley, V. R.; Dahlem, A. M., Dellinger, J. A.; Harlin, K. S.; Carmichael, W. W. *Toxicon* **1988**, *26*, 750.
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(5) Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. *Biochem. Pharm.* **1961**, *7*, 88. A modification of the assay described in this paper was used to observe the enzyme inhibitory activity. Solutions of samples to be tested were first spotted on filter paper or a TLC plate and sprayed with a mixture of acetylthiocholine (5 mg/mL) and 5,5'-dithiobis(2-nitrobenzoic acid) (5 mg/mL) in ethanol. After drying in a stream of air, a solution of electric eel acetylcholinesterase (EC 3.1.1.7, 0.5 units/mL) was next applied to the paper or plate. Active samples showed a white zone on a dense yellow background.

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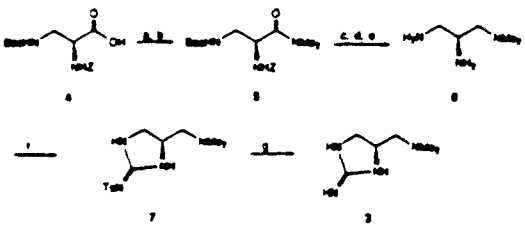
Scheme I[a]



[a]Reagents and conditions: (a) *N*-hydroxysuccinimide (1.2 equiv), DCC (1.2 equiv), dioxane, 0 °C 10 min → room temperature 15 h; (b) Me_2NH (2 equiv) in ether, room temperature 10 min; (c) CF_3CO_2H, room temperature 1 h; (d) 10% Pd–C, H_2, MeOH; (e) excess BH_3/Me_2S, THF, reflux 15 h; (f) *S,S*-dimethyl-*N*-tosylimidodithiocarbonimidate (1 equiv), EtOH, reflux 15 h; (g) 48% HBr, reflux 4 h.

relatively stable in neutral or acidic (pH 3–5) media.[6] Anatoxin-a(s) from cultured and field-collected *A. flos-aquae* exhibited identical chemical and spectral properties, including optical (CD in H_2O: $[\theta]_{207}$ −3300, $[\theta]_{232}$ +3900).

Mass spectral analysis of anatoxin-a(s) [positive FABMS (*m/z* 253.1067, MH^+), negative FABMS (*m/z* 251, $M-H^-$), FDMS (*m/z* 253, MH^+)] indicated the molecular formula $C_7H_{17}N_4O_4P$. The ^1H and ^{13}C NMR spectra[7,8] revealed the presence of dimethylamino and P-OMe (J_{H-P} 11.0 Hz; J_{C-P} 6.7 Hz) groups, a 1,2,3-trisubstituted propane unit, and an sp^2 carbon that was fully substituted by heteroatoms (δ_C 163.7). The methoxyl protons and carbon were the only ones showing distinct coupling to phosphorus. Only one signal was seen in the ^{31}P NMR spectrum and its chemical shift (δ 6.16) agreed well for either a phosphate ester or phosphoramide.[9] The J_{gem} (−10.1 Hz) and $^1J_{H,C}$ values for the protons in one of the methylenes of the propane unit suggested that this CH_2 was in a five-membered ring, along with the adjacent CH.

More information was obtained from NMR analysis of anatoxin-a(s) that had been uniformly enriched to 50% ^{13}C and 90+% ^{15}N[10,11] (See also Supplementary Material). The following conclusions could be made: (1) The sp^2 carbon at 163.7 ppm was connected to three nitrogens of a guanidine group and that two of these nitrogens were attached to the CH and CH_2 in the five-membered ring. (2) The NMe_2 group was connected to the side-chain CH_2 on the resulting imidazoline.[12] (3) No nitrogens were connected to the phosphorus; a methyl phosphate group was therefore present in the toxin. (4) The methyl phosphate group was attached to one of the nitrogens ($^2J_{P-N}$ 4 Hz); the toxin was therefore zwitterionic.

Anatoxin-a(s) slowly decomposed during storage at −20 °C into a mixture of **2**, **3** (sometimes), and monomethyl phosphate, separable by Toyopearl HW40F chromatography. Compound **2** (FABMS, MH^+ *m/z* 159.1245; CD in H_2O, $[\theta]_{235}$ +2400), which differed from compound **3** by an oxygen, could be converted into **3** (FABMS, MH^+ *m/z* 143.1298; CD in H_2O, $|\theta|_{198}$ +11 000) by catalytic hydrogenation (Pd–C/MeOH). Hydrolytic removal of the monomethyl phosphate group caused a diamagnetic shift of the H-5 signal from 4.71 ppm in **1** to 4.48 ppm in **2**; the methylene ^1H chemical shifts, however, were essentially identical for the two compounds. Although the ^1H chemical shifts for **3** were similar to those for **2**, except for one of the H-6 signals which was shifted upfield appreciably (−0.37 ppm), the ^{13}C chemical shifts were significantly different, i.e., upfield for C-5 (−7.8 ppm) and C-2 (−1.9 ppm) and downfield for C-4 (+2.7 ppm) and C-6 (+2.4 ppm).[13,14] These chemical shift differences were consistent with placements of the hydroxyl group on N-1 in **2**[15] and the methyl phosphate group on N-1 in **1**. Anatoxin-a(s) therefore had to have structure **1**.

To elucidate the absolute configuration at C-5, *R*- and *S*-**3** were prepared from D- and L-asparagine, respectively (Scheme I). N^2-(Benzyloxycarbonyl)-N^3-(*tert*-butoxycarbonyl)-L-2,3-diaminopropionic acid (**4**),[16] for example, was converted to dimethylamide **5** via the *N*-hydroxysuccinimide ester.[17] After removal of the amino-protecting groups (trifluoroacetic acid; H_2/Pd–C), the resulting diamine was reduced with BH_3–Me_2S complex[18] to give the triamine **6**, which was then treated with *S,S'*-dimethyl-*N*-tosylimidodithiocarbonimidate[19] to furnish the tosylguanidine **7**. Removal of the *N*-tosyl group was accomplished by refluxing **7** in 48% HBr.[20] Synthetic **3** showed identical chromatographic properties and ^1H and ^{13}C NMR spectra with the degradation product. The CD spectrum of **3** derived from anatoxin-a(s) was identical with that of synthetic **3** from L-Asn ($[\theta]_{198}$ +13 000), which meant that C-5 was *S*.

Anatoxin-a(s) is a unique phosphate ester of a cyclic *N*-hydroxyguanidine. The structure and reactivity is reminiscent of an ester of *N*-hydrosuccinimide or 1-hydroxybenzotriazole. Cholinesterase inactivation may proceed by nucleophilic attack of Ser at the esteratic site of the enzyme on the phosphate group of **1** with concomitant elimination of **2**.[21]

Acknowledgment. This research was supported by NSF Grant CHE-8800527 (R.E.M.) and in part by U.S. Army Medical Research Acquisition Activity, Contract No. 17-87-C-7019, Department of Army Medical Defense (W.W.C.). S.M. thanks the Naito Foundation for supplemental fellowship support. FABMS and MS–MS studies were carried out at the Midwest Center for Mass Spectrometry (M. L. Gross, director) by R. L. Cerny. Preliminary FABMS studies were conducted at the Institute of Applied Microbiology, University of Tokyo by H.

(6) Air evaporation of a methanolic solution of the toxin results in significant hydrolysis to **2**.

(7) All NMR spectra have been determined in D_2O with 5 μL of acetic acid-d_4 added per 0.5 mL of D_2O, unless otherwise noted.

(8) **1**: ^1H NMR δ 3.00 (s, NMe_2), 3.47 (dd, J = 2.9 and −13.9 Hz, H-6), 3.51 (dd, J = 9.7 and −10.1 Hz, H-4), 3.75 (dd, J = 9.3 and −13.9 Hz, H-6), 3.79 (d, $^3J_{H-P}$ = 11.0 Hz, P–OMe), 4.01 (dd, J = 9.4 and −10.1 Hz, H-4), 4.71 (m, H-5); exchangeable proton signals seen in acidic 80% H_2O/20% D_2O, δ 8.21 and 7.72 (two v br s, NH_2 on C-2), 7.44 (br s, H-3); ^{13}C NMR δ 43.9 (v br, NMe_2), 45.3 (C-4), 56.1 (d, $^3J_{C-P}$ = 6.7 Hz, P–OMe), 58.7 (C-6), 60.3 (C-5), 163.7 (C-2).

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(10) The labeled toxin was isolated from *A. flos-aquae* NRC 525-17 that had been grown in culture on $NaH^{13}CO_3$ (99 atom %) and $Na^{15}NO_3$ (99 atom %) by using the procedure described in the following: Moore, R. E., Bornemann, V., Niemczura, W. P.; Gregson, J. M.; Chen, J.-L., Norton, T. R., Patterson, G. M. L.; Helms, G. L. *J. Am. Chem. Soc.* **1989**, *111*, 6128.

(11) I uniformly enriched with ^{13}C to 50+% and ^{15}N to 90+%. ^1H NMR signals for NMe_2 and H-6 ($^1J_{H-C}$ = 144 Hz), OMe ($^1J_{H-C}$ = 148 Hz), H-4 and H-5 ($^1J_{H-C}$ = 151 Hz); ^{13}C NMR δ 43.9 (v br — doublet at 43.8 ppm in MeOH-d_4) or two doublets at 42.0 and 45.7 ppm in D_2O/CF_3CO_2H, J_{C-N} = 4.1 Hz, NMe_2), 45.3 (dd, J_{C4-C5} = 33.5 Hz, J_{C4-N1} = 8.7 Hz, J_{C4-N3} = 0, C-4), 56.1 (d, $^2J_{C-P}$ = 6.7 Hz, P–OMe), 58.7 (br d, J_{C5-C6} = 40.5 Hz, J_{C6-N3} = 2–3 Hz, C-6), 60.3 (br t, J_{C5-N1} = 0 Hz, C-5), 163.7 (td, J_{C2-N} = 23.5, 23.5, and 11.5 Hz, C-2); ^{15}N NMR (acidic 4:1 H_2O/D_2O) δ 350.6 (NMe_2), 306.9 and 306.7 (br, NH_2 on C-2 and N-4), 300.2 (N-1); ^{31}P NMR (acidic 4:1 H_2O/D_2O) δ 6.15 (d, $^3J_{P-N1}$ = 4 Hz).

(12) Also supported by FAB MS–MS and high-resolution data. Fragmentation of MH^+, for example, leads to *m/z* 58 (Me_2N^+=CH_2) as the base peak.

(13) **2**: ^1H NMR δ 3.00 (s, NMe_2), 3.46 (dd, J = 8.0 and −10.0 Hz, H-4), 3.47 (dd, J = 4.5 and −13.9 Hz, H-6), 3.75 (dd, J = 6.8 and −13.9 Hz, H-6), 3.93 (dd, J = 8.5 and −10.0 Hz, H-4), 4.48 (dddd, H-5); ^{13}C NMR δ 44.7 (q, NMe_2), 44.9 (t, C-4), 58.7 (t, C-6), 58.8 (d, C-5), 162.5 (s, C-2).

(14) **3**: ^1H NMR δ 2.96 (s, NMe_2), 3.38 (dd, J = 4.8 and −13.4 Hz, H-6), 3.48 (dd, J = 8.1 and −13.4 Hz, H-6), 3.50 (dd, J = 5.4 and −10.1 Hz, H-4), 3.98 (dd, J = 9.7 and −10.1 Hz, H-4), 4.55 (dddd, H-5); ^{13}C NMR δ 44.3 (q, NMe_2), 47.6 (t, C-4), 51.0 (d, C-5), 61.1 (t, C-6), 160.6 (s, C-2).

(15) When the hydroxyl group is removed from a *N*-hydroxyguanidine, the signals for the carbons α to the relevant nitrogen shift upfield, whereas the ones for the β-carbons shift downfield, e.g., neosaxitoxin and saxitoxin (Shimizu, Y.; Hsu, C., Fallon, W. E.; Ohtani, Y. *J. Am. Chem Soc.* **1978**, *100*, 6791) and 6-*N*-hydroxyarginine and arginine (Seto, H., Kovama, M., Ogino, H., Tsuruoka, T. *Tetrahedron Lett.* **1983**, *24*, 1805).

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Morisaki and S. Iwasaki. We thank P. Thorn (WSU) for assistance in toxin isolation, V. Bornemann and B. Moore (UH) for culturing *A. flos-aquae* in ^{13}C and ^{15}N enriched media, J. Stewart (UH) and N. A. Mahmood (WSU) for carrying out initial purification and characterization studies on **1** and **2**, and M. A. Tius (UH) for helpful discussions on the synthesis of **3**.

Supplementary Material Available: ^{1}H, ^{13}C, and ^{31}P NMR spectra of **1**, **2**, and **3**, ^{1}H, ^{13}C, ^{15}N, and ^{31}P NMR spectra of 50% ^{13}C and 90+% ^{15}N enriched **1**, and experimental details for the synthesis of *R*- and *S*-**3** from D- and L-Asn (16 pages). Ordering information is given on any current masthead page.

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